Syringolin A:

Biosynthesis, Regulation and Effects in Plants

Dissertation

zur Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

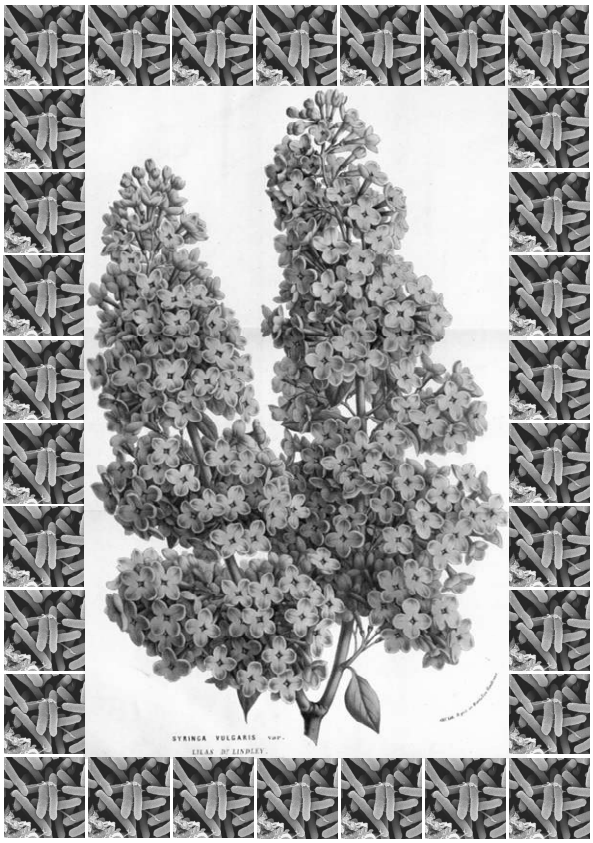
vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der Universität Zürich

von
Christina Ramel
von
Gretzenbach SO

Promotionskomitee
Prof. Dr. Leo Eberl (Vorsitz)
Prof. Dr. Robert Dudler (Leitung der Dissertation)
PD Dr. Cornelia Reimmann (Gutachterin)

Zürich 2012

Am Anfang war Staunen, Begeisterung und Freude an der Natur….



*

* *Syringa vulgaris* L. 'Dr. Lindley' from Flore des Serres, 1859.
Pseudomonas syringae pv. *syringae* B728a, Gordon Vrdoljak, Electron Microscopy Laboratory, U.C. Berkeley

TABLE OF CONTENTS

Summary

Syringolin A is a small-molecule virulence factor secreted by certain strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae.* The peptide derivative is exceptional with regard to its interesting chemical structure, complex regulation of its biosynthesis, and special mode of action.

Syringolin A is synthesised by a non-ribosomal peptide/polyketide synthetase which is encoded by five clustered genes, *sylA*-*sylE*. Syringolin A consists of a tripeptide part formed by a twelve-membered ring and an N-terminal valine that is joined to a second valine via a very unusual ureido group so far only known from a few natural products of cyanobacteria. Analysis of the syringolin A synthetase gene cluster led to a biosynthesis model that fully explained the biosynthesis of the tripeptide part but not the incorporation of the ureido group. The regulation of syringolin A biosynthesis appeared to be complex and occured *in vitro* only under specific *in planta*-mimicking conditions. Originally isolated because of its ability to induce aquired resistance against *Pyricularia oryzae* in rice, syringolin A's mode of action was shown to be the irreversible inhibition of the eukaryotic proteasome.

The objectives of this work were to explore the biology of syringolin A, including biosynthesis mechanisms, the understanding of its regulation and the molecular pathways by which syringolin A affects plant-pathogen interactions. Although the formation of the unique ureido linkage was hitherto unexplained, we demonstrated that the *sylA*-*sylE* genes were sufficient to direct syringolin A biosynthesis in heterologous organisms lacking a syringolin gene cluster. NMR analysis of syringolin A isolated from cultures grown in the presence of $NaH^{13}CO_3$ revealed the biosynthetic origin of the ureido group to be bicarbonate incorporated by a carbamylation reaction of valine, likely mediated by the *sylC* gene product. The insights into syringolin A's biosynthesis

are also of relevance to understand the biosynthesis of other bioactive ureiodo group-containing molecules.

It was known that syringolin A biosynthesis is controlled by the GacS/GacA two-component system and that the *sylA* gene codes for a HTH LuxR-type regulator. Here we report that syringolin A production is abolished in *sylA* deletion mutants and strongly enhanced by *sylA* overexpression. The conclusion that SylA is the transcriptional activator of syringolin biosynthesis was confirmed by the demonstration that SylA directly binds to, and activates *sylB and sylC* promoter regions, which were defined by overlapping deletion analysis using reporter constructs *in vitro* and *in planta*. The activity of the *sylA* gene in turn was shown to be dependent on SalA, another HTH LuxR-type transcription factor that itself is regulated by the GacS/GacA system. In addition, we demonstrated that syringolin A biosynthesis was independent of acyl-homoserine lactone-mediated quorum sensing regulation, but that its synthesis was increased by oxygen limitation.

The proteasome plays an essential role in many regulatory pathways. It is involved e.g. in hormone signalling, in responses to environmental changes and pathogen attack as well as in the establishment of disease immunity. Thus, inhibition of the plant's proteasome will result in pleiotropic effects, and it was not obvious which of these would be beneficial for the pathogen. Here we report that syringolin A-producing bacteria are able to counteract stomatal immunity and that it is the proteasome inhibitory capacity of syringolin A that results in suppression of salicylic acid defense signalling, thereby counteracting the stomatal immune response and salicylic acid-dependent gene activation.

Zusammenfassung

Syringolin A ist ein kleinmolekularer Virulenzfaktor, welcher von einigen Stämmen des phytopathogenen Bakteriums *Pseudomonas syringae* pv. *syringae* sezerniert wird. Das Peptidderivat ist aussergewöhnlich in Bezug auf seine chemische Struktur, die komplexe Regulation seiner Biosynthese, sowie bezüglich seines speziellen Wirkmechanismus. Syringolin A wird von einer nicht-ribosomalen Peptidsynthetase synthetisiert, welche von den fünf geclusterten Genen *sylA – sylE* kodiert wird. Die Verbindung besteht aus einem Tripeptidteil, der sich aus einem 12-gliedrigen Ring und einem N-terminalen Valin zusammensetzt, welches seinerseits durch eine sehr ungewöhnliche Ureido-Gruppe mit einem zweiten Valin verknüpft ist. Solche Ureido-Gruppen kennt man bisher nur von wenigen natürlichen Produkten von Cyanobakterien. Die Analyse des Syringolin A-Synthetase-Genclusters hat zur Postulierung eines Biosynthese-Modells geführt, welches die Synthese des Tripeptidteils vollständig erklärt, jedoch nicht die Bildung der Ureido-Gruppe. Die Regulation der Syringolin A-Biosynthese schien komplex zu sein und in *in vitro* Kulturen nur unter spezifischen, *in planta*-ähnlichen Bedingungen zu erfolgen. Syringolin A wurde ursprünglich aufgrund seiner Fähigkeit, in Reis eine erworbene Resistenz gegen *Pyricularia oryzae* zu induzieren, isoliert. Später konnte gezeigt werden, dass der Wirkmechanismus von Syringolin A die irreversible Inhibition des eukaryotischen Proteasoms ist.

Die Ziele dieser Arbeit waren Untersuchungen zur Biologie von Syringolin A, zu seinen Biosynthese-Mechanismen, zum Verständnis der Biosynthese-Regulation, sowie zu den molekularen Vorgängen, mittels derer Syringolin A Pflanzen-Pathogen-Interaktionen beeinflusst. Obwohl die Bildung der besonderen Ureido-Gruppe bis anhin ungeklärt war, konnten wir zeigen, dass die *sylA – sylE*-Gene ausreichend sind, um die

Biosynthese von Syringolin A in heterologen Organismen, die keinen Syringolin-Gen-Cluster besitzen, zu erreichen. Die NMR-Analyse von Syringolin A, das aus mit $NaH^{13}CO_3$ versetzten Kulturen isoliert wurde, zeigte, dass die biosynthetische Herkunft der Ureido-Gruppe Bicarbonat ist. Dieses wird durch eine vermutlich durch das *sylC*-Gen-Produkt katalysierte Carbamylierungsreaktion von Valin eingefügt. Die gewonnenen Erkenntnisse betreffend der Syringolin A-Biosynthese tragen auch zum Verständnis der Biosynthese anderer bioaktiver Moleküle bei, welche ebenfalls Ureido-Gruppen enthalten.

Es war bekannt, dass die Syringolin A-Biosynthese durch das GacS/GacA Zwei-Komponenten-System kontrolliert wird, sowie dass das *sylA*-Gen einen HTH-LuxR-Typ-Regulator kodiert. Hier zeigen wir, dass eine *sylA*-Deletions-Mutante kein Syringolin A produzierte und dass die Syringolin A-Produktion durch eine *sylA*-Überexpression stark erhöht wurde. Die Schlussfolgerung, dass SylA der Transkriptions-Aktivator der Syringolin-Biosynthese ist, konnte durch Resultate bestätigt werden, die zeigen, dass SylA direkt an *sylB*- und *sylC*- Promotorregionen bindet und diese aktiviert. Diese Promotorregionen wurden durch überlappende Deletions-Analysen mittels Reporterkonstrukten *in vitro* und *in planta* definiert. Die Aktivität des *sylA*-Gens wiederum zeigte sich abhängig von SalA, einem weiteren HTH-LuxR-Typ- Transkriptionsfaktor, welcher seinerseits durch das GacS/GacA System reguliert wird. Des Weiteren zeigten wir, dass die Syringolin A-Biosynthese unabhängig von der Quorum Sensing-Regulation ist, jedoch durch Sauerstoff-Limitation erhöht wird.

Das Proteasom spielt bei Pflanzen eine wesentliche Rolle in vielen regulatorischen Abläufen. Es ist beispielsweise involviert in der Hormon-Signaltransduktion, in Reaktionen sowohl auf Umweltveränderungen als auch auf Angriffe durch Pathogene, sowie in der Etablierung der Immunität gegen Krankheiten. Demzufolge führt die Inhibierung des pflanzlichen Proteasoms zu pleiotropen Effekten,

wobei nicht klar war, welche darunter für das Pathogen vorteilhaft sind. Hier zeigen wir, dass Syringolin A-produzierende Bakterien fähig sind, die stomatale Immunität zu unterdrücken, und dass es die Proteasom-inhibierende Eigenschaft von Syringolin A ist, welche den Salicylsäure-Abwehr-Signalweg unterdrückt und auf diese Weise der Stomata-Immunantwort und der Salicylsäure-abhängigen Gen-Aktivierung entgegen-wirkt.

1.

GENERAL INTRODUCTION

1.1 Plant-pathogen interactions

1.1.1 The two-layered innate immune system of plants

Plants constantly interact with both beneficial and antagonistic organisms. On one hand, plants have to accommodate beneficial organisms and on the other hand, they have to effectively counteract harmful ones. To recognise the organisms and to respond in the appropriate, either conducive or defensive manner, plants have evolved highly sophisticated strategies (Pieterse and Dicke, 2007).

To perceive and combat pathogen invasions, plants evolved an innate immune system with two layers (Jones and Dangl, 2006; Segonzac and Zipfel, 2011) (Figure 1). The first comprises the extracellular recognition of highly conserved, unspecific elicitor molecules such as lipopolysaccharide or flagellin. These so-called pathogen- or microbe-associated molecular patterns (PAMPS or MAMPS) are perceived through transmembrane pattern recognition receptors (PRRs). The activation of PRRs by PAMPs evokes a variety of cellular responses, some of which result in PAMP-triggered immunity (PTI).

To subvert this basal immunity, pathogens have evolved effector proteins (virulence factors) which are injected -often via the type-III secretion system- into the plant cell. The successful suppression of PTI responses by means of these effectors results in effector-triggered susceptibility (ETS).

To counter ETS, plants have evolved the second layer of the innate immune system, the so-called effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is triggered upon direct or indirect recognition of particular effector proteins by resistance gene (R-gene) products, many of which contain a leucin rich repeat domain. ETI is race-specific and follows the gene-for-gene concept (Flor, 1971). ETI often leads to the hypersensitive reaction (HR), which is characterized by localised programmed cell death. This cell death is associated with the synthesis and accumulation of the defense hormone salicylic acid (SA) in both local and systemic tissues and results in the induction of systemic acquired resistance (SAR) (Dong, 2001). PTI and ETI together can be seen as an overarching principle to perceive danger signals (Boller and Felix, 2009) (Figure 1).

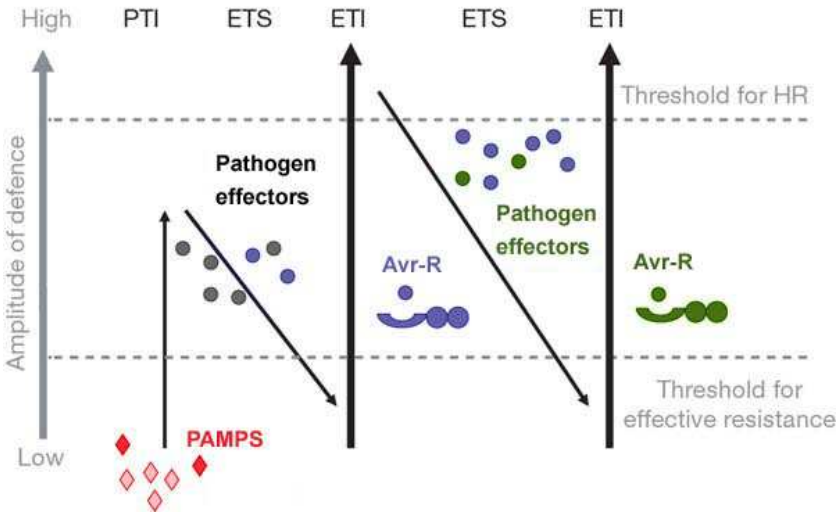


Figure 1. Zig-zag model of the two layered innate immune system.

The first layer of active plant defense comprises the extracellular recognition of unspecific pathogen-associated molecular patterns (PAMPS, in red) via pattern recognition receptors, leading to PAMP-triggered immunity (PTI). Successful pathogens are able to suppress PTI response by means of secreted effectors (grey), resulting in effector-triggered susceptibility (ETS).

During evolution, plants have responded through the development of cytoplasmic R proteins that recognize single effectors (indicated in blue). By means of this second layer of defense, effector-triggered immunity is activated (ETI). Upon selection pressure, pathogens either loose or alter the recognized effectors or additionally gain new ones to suppress the ETI response. In turn, new plant receptors evolve which recognize the new effectors (green), resulting again in ETI. This coevolution continues, with ongoing selection for novel pathogen isolates that defeat ETI and new plant genotypes that resurrect ETI. The diagram is adapted from Jones and Dangl (2006).

1.1.2 Stomatal immunity

The first step for a pathogen to colonise the apoplastic intercellular spaces is to successfully ingress into the plant tissue. Bacteria -unlike fungal pathogens- are unable to directly penetrate the plant epidermis. Therefore, bacteria use wounds, hydathodes, or stomata for entry. Stomata are not only passive entry portals but can be actively closed in response to potential pathogens or their effector molecules (Melotto et al., 2006). For example, fungal elicitors such as oligogalacturonic acid and chitosan are known to promote stomatal closure (Klusener et al., 2002; Lee et al., 1999). PRRs localised in the plasma membrane of guard cells perceive PAMPs and trigger the closure of stomata as a part of PTI, thereby hampering the entry of microbes (Melotto et al., 2008; Melotto et al., 2006).

In this stomatal immune reaction, several signalling components, e.g. absisic acid, nitric oxygen and the regulation of K^+ channels, are shared with the regulation of stomata by abiotic signals such as light and humidity (Zhang et al., 2008). Besides these common signalling components, mitogen-activated protein (MAP) kinase 3 and SA were found to be involved in PAMP-triggered stomatal closure (Melotto et al., 2006).

To counteract the stomatal immunity, pathogenic bacteria have evolved specific effectors to suppress the closure of stomata and thereby increasing the invasion of leaf tissue by bactaria. Examples of virulence factors actively inducing the re-opening of plant stomata are coronatine (Melotto et al., 2006) and a factor of unknown identity secreted by *Xanthomonas campestris* (Gudesblat et al., 2009).

1.2 *Pseudomonas syringae* pv. *syringae*

Pseudomonas syringae is a phytopathogenic γ-proteobacterium that colonises either the surface of the plant epidermis as an epiphyte or the intercellular spaces. *Pseudomonas syringae* is rod-shaped, has polar flagella, is strictly aerobic, and produces mostly fluorescent pigments. *P. syringae* is oxidase- and arginine-dihydrolase-negative, which distinguishes it from most other fluorescent pseudomonads. Although initially isolated from a diseased lilac tree (*Syringa vulgaris* L.) and therefore named *P. syringae* (Young, 1991), the species can be found on a wide range of plants in most regions of the world. Within the species, a large variety of specialisations of individual strains with respect to colonised plants occurs. Based on nutritional, biochemical, physiological, and DNA-based tests as well as on different pathogenic abilities, bacteria within the species were grouped into about 50 different pathovars. Among most of these pathovars, which exhibit rather narrow host ranges, the pathovar *syringae* is an exception. *Pseudomonas syringae* pv. *syringae* (*Pss*) can cause disease in over 180 plant species belonging to several unrelated genera (Bradbury, 1986).

Pss causes bacterial canker and blast of stone fruit trees (various *Prunus* species), brown spot and bacterial blight in pea (*Pisum sativum* L.) and bean (*Phaseolus vulgaris* L.) as well as stem canker and dieback of olives (*Olea europaea* L.). Furthermore, *Pss* causes for example blight in wheat, red streak in sugarcane (Rahimian, 1995), and bacterial apical necrosis of mango (*Mangifera indica* L.) (Cazorla et al., 1998). Its global occurrence makes *Pss* to one of the most important plant pathogens worldwide with deleterious effects on productivity of economically important plants (Ashorpour et al., 2008; Little et al., 1998; Martin-Sanz et al., 2011; Young, 1991).

Phytopatogenic bacteria harbour a conserved gene cluster, the *hrp* region, encompassing genes for the regulation and biosynthesis of a type-III secretion system required for pathogenicity on plant hosts (Salmond, 1994). The virulence proteins secreted by this pathway are responsible for the induction of the hypersensitive response in nonhost plants, pathogenicity in host plants and the ability to grow as epiphytes or endophytes of plants (Gopalan et al., 1996). In addition to type III effector proteins, *P. syringae* is reported to produce a wide spectrum of small molecules that can function as toxins or virulence factors. Most of these small molecules are the products of non-ribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) (Meier and Burkart, 2009). Several pathovars of *P. syringae* produce for example the polyketide toxin coronatine (COR), which is a molecular mimic of the plant hormone jasmonic acid (Ile-JA) and counteracts, among other effects in various plant hosts, stomatal immunity in Arabidopsis (Melotto et al., 2006).

Most strains of *Pss* characteristically produce two classes of lipodepsipeptide toxins, of which syringomycin and syringopeptin encoded by the syringomycin (*syr*) and syringopeptin (*syp*) genomic island are the most important ones. These key virulence determinants of *Pss* induce necrosis in plant tissues by forming pores in the host plasma membrane that leads to electrolyte leakage (Backman and Devay, 1971; Bender et al., 1999; Gross, 1991). Furthermore, certain strains of *Pss* were found to secrete the small-molecule virulence factor syringolin A, which is the focus of this thesis. In the research forming the basis of this work, the *Pss* strains B301D-R (isolated from pear) and B728a (a pathogen of bean) were mainly used.

1.3 Syringolin A

1.3.1 Discovery of syringolin

Syringolin A was discovered in the course of studying acquired resistance responses in rice (Reimmann et al., 1995). Infiltration of the non-host pathogen *Pss* into the leaves of rice plants triggered acquired resistance of rice to *Pyricularia oryzae*, the causing agent of rice blast (Smith and Métraux, 1991). One of the defence-related transcripts found to accumulate concomitantly with resistance induction was *pir7b*, which encoded an esterase of the α/β- hydrolase fold structural superfamily (Wäspi et al., 1998b). The fact that *pir7b* transcript accumulation only occurred after infiltration with certain *Pss* strains but not with others, as well as the finding that *pir7b* transcript accumulation was dependent on the bacterial two-component regulatory system GacS/GacA, indicated that *pir7b* mRNA accumulation was a specific response to an elicitor of the infiltrated bacteria (Reimmann et al., 1995). Subsequently, this elicitor was isolated, its structure elucidated and referred to as syringolin A (Wäspi et al., 1998a).

1.3.2 Structure and biosynthesis of syringolin A

Syringolins are secreted by strains of the phytopathogenic bacterium *Pss in planta* and under suitable culture conditions (Wäspi et al., 1998a; Wäspi et al., 1999). Special noteworthy structural features of syringolin A are a twelve-membered macrolactam ring containing two nonproteinogenic amino acids and an N-terminal acylation of valine to an additional valine via a very unusual ureido linkage (Wäspi et al., 1998a) (Figure 2).

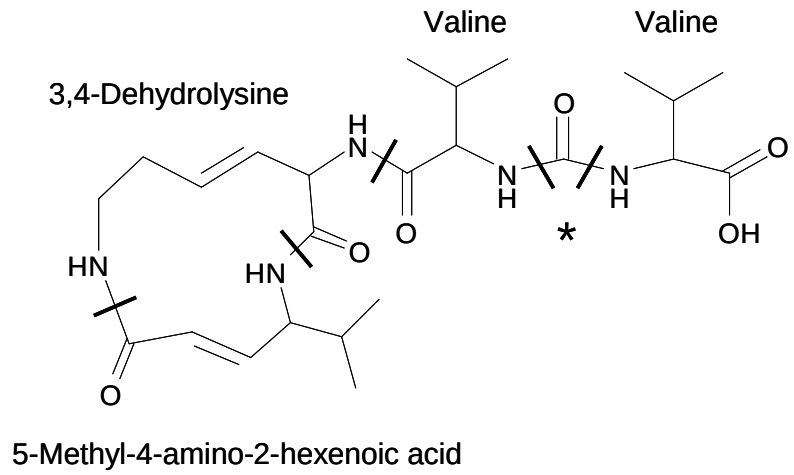


Figure 2. Structure of syringolin A.

Structure of syringolin A. The amino acids constituents of syringolin A are delimited by thick bars and labelled above and below the structure. The ureido group is indicated with an asterisk.

To direct syringolin biosynthesis, a gene cluster comprising the five genes *sylA*-*sylE* was shown to be necessary (Amrein et al., 2004) (Figure 3). The *sylA* gene encodes a protein with a HTH LuxR DNA-binding domain, suggesting that SylA is a transcriptional activator of the syringolin synthetase genes. The *sylB* gene product is homologous to a fatty acid desaturase and most likely responsible for the introduction of the double bond in the 3,4-dehydrolysine residue. The *sylC* and *sylD* genes encode nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules, and *sylE* encodes the putative syringolin exporter (Amrein et al., 2004).

Figure 3. Arrangement of the syringolin A synthetase genes *sylA* - *sylE*.

Boxes above and below the line denote ORFs on the top and the bottom strand, respectively.

NRPS are large multifunctional bacterial and fungal enzymes that are able to catalyse the synthesis of bioactive peptides of huge structural diversity according to the thiotemplate mechanism (Stachelhaus and Marahiel, 1995). Products include antibiotics, enzyme inhibitors, toxins and immunosuppressants. One of the largest bacterial NRPS known, comprising a total of 8 modules, is the *Pss* syringomycin synthetase SyrE (Guenzi et al., 1998). Many nonribosomally synthesised peptides are clearly distinguishable from ribosomally produced molecules by their unusual features, such as nonproteinogenic or modified amino acids, fatty acids of varying length, or heterocyclic rings (Finking and Marahiel, 2004). As the ribosomal protein production, the nonribosomal machinery requires at least three enzymatic activities: one for substrate activation, one for substrate carriage and one for peptide bond formation. The nonribosomal system comprises modules with an adenylation domain (A-domain) responsible for amino acid selection and activation, a peptidyl carrier protein (PCP) as the transport unit and a condensation domain (C-domain) for peptide bond formation. In addition, tailoring enzymes can be involved in the maturation of the NRPS-product. The NRPS domains are arranged in modules whose function is related to their structure and thereby can be subdivided into initiation modules without a C-domain and elongation modules containing a C-domain responsible for peptide bond formation between amino acyl substrates bound to adjacent modules (Stachelhaus et al., 1998). The growing peptide is handed over from one module to the next until it reaches the PCP-domain of the final module that usually contains a thioesterase (TE) domain responsible for the liberation of the product.

Type I PKS are also modular enzymes that, in analogy to fatty acid synthesis, extend a starter molecule by condensation/decarboxylation of malonate extender units. A typical PKS module as it is part of SylD consists of the three main domains acyl transferase (AT), acyl carrier protein (ACP) and keto synthase (KS) as well as of

optional domains, such as e.g. ketoacyl-ACP reductase (KR), β-hydroxyacyl-ACP dehydratase (DH) and enoyl-ACP reductase (ER) domains (Hopwood 1997).

The analysis of the structure and architecture of the *syl* gene cluster led to the postulation of a model that completely explains the biosynthesis of the tripeptide part of syringolin A (Amrein et al., 2004). According to the model (see chapter 2, Figure 1C), the first NRPS module of SylD is predicted to activate lysine, which is oxidized to 3,4-dehydrolysine by the *sylB* gene product. The second module is thought to activate valine, from which the 5-methyl-4-amino-2-hexenoic acid of the ring structure is synthesised by the PKS module, which extends the valine residue by a C_2 unit resulting from the condensation/decarboxylation of an activated malonyl moiety. The resulting diketide is reduced twice by the β-ketoreductase (KR) and the dehydratase (DH) domains to yield the double bond in the 5-methyl-4-amino-2-hexenoic acid moiety. The generation of the ureido group and its attached second valine which the model did not account for is the subject of chapter 2. In syringolin A biosynthesis, the TE domain of the PKS module of SylD is responsible for the release and ring formation between the terminal carboxyl group and the ε- amino group of the 3,4-dehydrolysine (Amrein et al., 2004) (chapter 2, Figure 1C).

Syringolin A is the predominant member of a family of closely related peptide derivatives which include syringolin A to syringolin F. The syringolin variants are all synthesised by the same peptide synthetase and differ from each other by amino acid substitutions or modifications, i.e. by the substitution of 3,4-dehydrolysine by lysine in the ring structure (syringolin B), the substitution of either one or both of the two valine residues in the linear tail part by isoleucine (syringolin C, D, and F, respectively), and a combination of the two types of substitutions (syringolin E) (Wäspi et al., 1999).

1.3.3 Structural analogues of syringolin A and other small molecules

The compounds structurally most similar to syringolins are the glidobactins, which are secreted by an unknown species belonging to the *Burkholderiales* (β-proteobacteria). Syringolins and glidobactins together constitute a structural class named syrbactins (Schellenberg et al., 2007). Glidobactins are acylated cyclic tripeptides of which the major variant is glidobactin A (Oka et al., 1988a; Oka et al., 1988b). The ring part of glidobactin A consists of the two nonproteinogenic amino acids erythro-4-hydroxylysine and 4-amino-2-pentenoic acid synthesised in analogy to syringolin A by a partly conserved gene cluster (Schellenberg et al., 2007). Functional homologues of the syringolin/glidobactin gene clusters are found in the human pathogens *Burkholderia pseudomallei* (the causative agent of melioidosis), *B. oklahomensis* and *Photorhabdus asymbiotica* as well as in the insect pathogen *Photorhabdus luminescens*. These organisms are therefore hypothesised to synthesise syrbactin-like compounds (Schellenberg et al., 2007).

Natural products known to share with syringolin A the ureido moiety, are some small bioactive molecules, mostly secondary metabolites from cyanobacteria, such as e.g. anabaenapeptins and brunsvicamides (Walther et al., 2009). These so called anabaenapeptin-like molecules show a very interesting broad spectrum of biological activities (Murakami et al., 1997; Okumura et al., 2009).

1.3.4 Effects of syringolin A on plants and mode of action

Syringolin A was shown to be one of the determinants by which nonhost rice plants are able to recognise *Pss*, leading to acquired resistance of rice against *Pyricularia oryzae* (Wäspi et al., 1998b). In addition, syringolin A has been shown to induce

hypersensitive cell death of powdery mildew-colonised wheat and Arabidopsis cells and thus, to reprogram a compatible interaction into an incompatible one (Wäspi et al., 2001). Syringolin A itself does not show phytotoxic, antibiotic or fungitoxic effects (Wäspi et al., 1998a; Wäspi et al., 2001), but it could be demonstrated that syringolin A is a virulence factor in the interaction of *Pss* strain B728a with its host *Phaseolis vulgaris* (Schellenberg et al., 2007).

In order to elucidate the mode of action of syringolin A, the transcriptional changes accompanying the HR triggered upon syringolin A spraying of powdery mildew-infected wheat and Arabidopsis plants were monitored. This transcriptome change analysis revealed an accumulation of transcripts encoding all proteasome subunits as well as of many heat-shock proteins (Michel et al., 2006). Because this effect is known to be an indicator for proteasome inhibition (Fleming et al., 2002; Meiners et al., 2003), it was tested whether syringolin A was a proteasome inhibitor. *In vitro* experiments showed that syringolin A acts indeed as an irreversible inhibitor of all three catalytic activities of the eukaryotic proteasome. Because they act via a novel mechanism, syringolins (together with the glidobactins), form a new structural class of proteasome inhibitors (Groll et al., 2008; Schellenberg et al., 2007)

The ubiquitin–proteasome degradation pathway was shown to be essential for pathogen defence and disease immunity of plants (Dong et al., 2006; Goritschnig et al., 2007). Thus, and this is the subject of chapter 4, *Pss* was suggested to suppress hosts defence reactions by inhibition of the host proteasome using syringolin A.

1.4 Regulation of syringolin A biosynthesis

1.4.1 The GacS/GacA two-component regulatory system

Syringolin A biosynthesis was shown to depend on a functional GacS/GacA two-component regulatory system (Reimmann et al., 1995; Wäspi et al., 1998b). The GacS/GacA two-component system plays an important role in a large variety of Gram-negative bacteria. In response to environmental signals, this regulatory system helps e.g. phytopathogenic bacteria to adapt to fluctuating physicochemical conditions characteristic of the phyllosphere, as well as to colonise their specific ecological niches. Controlling, among other traits, the production of extracellular products, ecological fitness, stress tolerance and virulence, the GacS/GacA two-component system is suggested to mediate a switch between primary and secondary metabolism (Heeb and Haas, 2001).

Environmental signals still unknown trigger the activation of the sensor kinase GacS (first described in *Pss* strain B728a as lesion manifestation factor LemA (Hrabak and Willis, 1992)), which then undergoes autophosphorylation. By phosphotransfer, the response regulator GacA (first described in *P*. *fluorescens* as a global activator of antibiotic and cyanide production (Laville et al., 1992)) is activated, which then leads to the expression of target genes. Because the periplasmatic loop domain of GacS is poorly conserved in diverse bacteria, a common signal interacting with this domain is unexpected (Heeb and Haas, 2001). For more details about the function of GacS/GacA see Dutta (Dutta et al., 1999; Perraud et al., 2000). Coding target genes directly recognised and controlled by GacA are not known, but the major effect of the GacS/GacA system seems to be at the posttranscriptional level and involves small RNAs and RNA binding proteins (Heeb and Haas, 2001; Lapouge et al., 2008).

Apart from syringolin A biosynthesis, many processes relevant for pathogenesis of *Pss* are regulated by the GacS/GacA two-component regulatory system. This system is required for lesion formation on bean leaves, for the control of the biosynthesis of the toxins syringomycin and syringopeptin as well as of N-acyl-homserine-lactones, the signaling molecules of the quorum sensing system, and of alginate and exoprotease (Heeb and Haas, 2001; Hrabak and Willis, 1992; Kitten et al., 1998; Rich et al., 1992; Willis et al., 1990). With regard to syringolin A biosynthesis, the GacS/GacA systems may well be a cause of the fact that syringolin A production *in vitro* requires still culture conditions in a minimal medium originally optimized for syringomycin production (Gross, 1985). This minimal medium (dubbed SRM_{AF}-medium) contains iron and the plant signal molecules arbutin and D-fructose (Mo and Gross, 1991b).

1.4.2 Quorum sensing

The fact that syringolin A production *in vitro* requires still culture conditions, in which much lower cell densities are reached than in shaken, well aerated cultures, and the fact mentioned above that the GacS/GacA system also controls the quorum sensing (QS) system, made it seem not unlikely that syringolin A production might be negatively regulated by QS.

QS is the population density dependent intercellular communication system of bacteria, which facilitates the adaptation to changing environmental conditions. As a function of cell density, the concentration of chemical signal molecules (so called autoinducers) increases and reaches a threshold concentration which results in altered gene expression and profound physiological changes (for reviews see Miller and Bassler, 2001; Whitehead et al., 2001). QS signal transduction mechanisms are used

by Gram-negative and Gram-positive bacteria to coordinate a great variety of physiological processes including bioluminescence, biofilm formation, surface motility, sporulation, symbiosis, expression of virulence genes and the production of exoenzymes and antibiotics. Many types of microbially derived signalling molecules have been described. In general, Gram-positive bacteria use processed oligo-peptides (Kleerebezem et al., 1997) and Gram-negative use fatty acid derivatives, mostly acylated homoserine lactones (HSL). Most of the QS circuits identified in Gram-negative bacteria contain homologues of the LuxI autoinducer synthetase and LuxR regulator proteins of *V. fischeri* (Miller and Bassler, 2001). The LuxI-type inducer enzymes synthesise HSL, which diffuses then from the cytoplasm across the cell membrane. When the HSL concentrations, which are proportional to the population of producing cells, reach a certain threshold, HSL is bound by the binding site of the N-terminal domain of the LuxR-type receptors (Engebrecht and Silverman, 1984). The binding activates the LuxR-type receptor, whose helix-turn-helix (HTH) DNA binding domain located in the C-terminal part binds to recognition sequences (lux-like boxes) in the promoter regions of the genes which it controls (Churchill and Chen, 2011).

In *Pss*, cell-to-cell communication is mediated by *N*-acyl homoserine lactones (AHLs), more precisely by 3-oxo-C6-HSL. In *Pss*, QS was shown to coordinate traits important in the interaction with host plants. In strain B728a, QS was found to regulate e.g. the expression of genes responsible for extracellular polysaccharide production and motility as well as for factors contributing to virulence (Dulla and Lindow, 2009; Quinones et al., 2005). The *Pss* QS system consists of the AHL synthase AhlI and the AHL regulator AhlR (Dumenyo et al., 1998; Quinones et al., 2004). The production of the 3-oxo-C6-HSL signal is catalysed by AhlI, whereas AhlR forms a stable complex with the AHLs and subsequently activates the transcription of *ahlI,* which leads to increasing AHL concentrations with increasing cell density (Quinones et al., 2004). For *ahlI* transcription, the regulator AefR was shown to be required in addition to the

AhlI/AhlR system. As mentioned above, GacA was found to activate the quorum sensing system via an independent pathway (Quinones et al., 2004).

1.4.3 SylA and other HTH LuxR-type transcription factors

As mentioned above, the product of the *sylA* gene exhibits a HTH domain, which represents a prevalent sub-structure in DNA-binding proteins occurring in a variety of organisms (Pabo and Sauer, 1992). Based on sequence and structural similarities, HTH-containing regulators can be divided into more than 10 groups, one of them being similar to the QS regulator LuxR. SylA belongs to this group of LuxR-type regulators. With regard to the N-terminus, the LuxR-type proteins can be grouped into two major subfamilies: The response regulator subfamily containing the characteristic acid pocket of receiver domains, and the autoinducer-binding regulator subgroup containing seven highly conserved residues. SylA lacks both of these domains, like SalA, SyrF, and SyrG, which represent a subfamily of the LuxR-type of regulators (Lu et al., 2002a). As the *sylA* gene was required for syringolin A biosynthesis, it was hypothesized that its product is a transcriptional activator of other syringolin A synthetase genes (Amrein et al., 2004).

The SalA, SyrF and SyrG proteins are encoded in the syringopeptin and syringomycin synthetase gene clusters (Lu et al., 2002a; Wang et al., 2006). SalA was characterised as a member of the GacS/GacA two-component signal transduction system (Kitten et al., 1998). SalA was shown to control the expression of genes involved in the synthesis, secretion, and regulation of both syringomycin (syr) and syringopeptin (syp) phytotoxins. Thus, it represents a link between the GacS/GacA regulatory system and pathogenicity (Lu et al., 2005). Among the SalA-regulated members of the *syr-syp* genomic island are *syrF* and *syrG*, which are homologues of

SalA and themselves members of the LuxR-type family of regulatory proteins (Lu et al., 2002a; Lu et al., 2005). *SyrF* is required for syringomycin production, and the *syrG* gene is not essential for toxin production but associated with virulence of *P*. *syringae* pv. *syringae*. Virulence genes associated with Hrp type-III secretion were found to be SalA- independent (Lu et al., 2005).

1.5 Aims of the thesis

The aims of this thesis were explorations of the biology of syringolin A, including the biosynthesis mechanisms, the understanding of its regulation and the molecular pathways by which syringolin A affects plant-pathogen interactions.

In detail, the objectives were:

- to demonstrate that the heterologous expression of the *sylA-sylE* gene cluster enables the biosynthesis of *bona fide* syringolin A.
- to explain the biosynthesis of syringolin A's unusual ureido group.
- to elucidate the regulation of syringolin A biosynthesis, including the determination of promotor regions and the identification of transcriptional activators.
- to better understand the role of syringolin A in the counteraction of stomatal innate immunity.

The approaches undertaken towards these aims form the content of chapters 2, 3 and 4.

In chapter 2 we demonstrated that, apart of the *sylA*-*sylE* genes, no additional syringolin A specific genes were needed to achieve biosynthesis of the ureido group joining the two valine residues. We could show by NMR analysis of syringolin A isolated from cultures grown in the presence of $NaH^{13}CO_3$ that the source of the ureido carbonyl group is bicarbonate or carbon dioxide. We further hypothesised that this carbonyl group is incorporated by carbamylation of valine mediated by the *sylC* gene product (Ramel et al., 2009).

Chapter 3 is aimed at the elucidation of the regulation of syringolin A biosynthesis. We demonstrated that the *sylC, sylD, and sylE* genes form an operon transcribed by promoter sequences located between the *sylC* and the *sylB* gene. By assays with *sylB* and *sylC* promoter fragments translationally fused to the *lacZ* gene, promoter sequences required for *syl*-gene activity both *in vitro* and *in planta* were defined. Further, we could show that the HTH LuxR-type transcription factor encoded by the *sylA* gene is activating the *sylB* and *sylC* promotor and that SylA is binding directly to these promoters. In addition, we demonstrated that syringolin A biosynthesis is dependent on a functional *salA* gene but not on the HSL quorum sensing system, and we present evidence that oxygen concentration plays a role (Ramel et al. 2012, submitted).

In chapter 4, it is presented how syringolin A counteracts stomatal immunity by proteasome inhibition. We report that in bean and *Arabidopsis*, syringolin A producing *Pss* B728a bacteria are able to re-open stomata and thus, counteract stomatal innate immunity. We demonstrated that proteasome activity is crucial for stomatal closure and guard cell function and we showed that bacteria-induced stomatal closure required SA and depended on NPR1, the key regulator of the SA defense pathway (Schellenberg et al.,2010).

2.

BIOSYNTHESIS OF THE PROTEASOME INHIBITOR SYRINGOLIN A: THE UREIDO GROUP JOINING TWO AMINO ACIDS ORIGINATES FROM BICARBONATE

Christina Ramel[1], Micha Tobler[1], Martin Meyer[1], Laurent Bigler[2], Marc-Olivier Ebert[3], Barbara Schellenberg[1], and Robert Dudler[1§]

BMC Biochemistry 2009

10:26

10:26 doi:10.1186/1471-2091-10-26

Published: 28 October 2009

[1] Institute of Plant Biology, University of Zurich, Zurich, Switzerland

[2] Institute of Organic Chemistry, University of Zurich, Zurich, Switzerland

[3] Laboratory of Organic Chemistry, ETH Zurich, Zurich, Switzerland

[§] Corresponding author

2.1 Abstract

Background

Syringolin A, an important virulence factor in the interaction of the phytopathogenic bacterium *Pseudomonas syringae pv. syringae B728a with its host plant Phaseolus* vulgaris (bean), was recently shown to irreversibly inhibit eukaryotic proteasomes by a novel mechanism. Syringolin A is synthesized by a mixed non-ribosomal peptide synthetase/polyketide synthetase and consists of a tripeptide part including a twelve-membered ring with an N-terminal valine that is joined to a second valine via a very unusual ureido group. Analysis of sequence and architecture of the syringolin A synthetase gene cluster with the five open reading frames *sylA-sylE* allowed to formulate a biosynthesis model that explained all structural features of the tripeptide part of syringolin A but left the biosynthesis of the unusual ureido group unaccounted for.

Results

We have cloned a 22 kb genomic fragment containing the *sylA-sylE* gene cluster but no other complete gene into the broad host range cosmid pLAFR3. Transfer of the recombinant cosmid into *Pseudomonas putida* and *P. syringae* pv. *syringae* SM was sufficient to direct the biosynthesis of bona fide syringolin A in these heterologous organisms whose genomes do not contain homologous genes. NMR analysis of syringolin A isolated from cultures grown in the presence of $NaH^{13}CO_3$ revealed preferential ^{13}C-labeling at the ureido carbonyl position.

Conclusions

The results show that no additional syringolin A-specific genes were needed for the biosynthesis of the enigmatic ureido group joining two amino acids. They reveal the source of the ureido carbonyl group to be bicarbonate/carbon dioxide, which we hypothesize is incorporated by carbamylation of valine mediated by the *sylC* gene product(s). A similar mechanism may also play a role in the biosynthesis of other ureido-group-containing NRPS products known largely from cyanobacteria.

2.2 Background

Syringolins are a family of closely related cyclic peptide derivatives that are secreted by many strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* (*Pss) in planta* and under certain culture conditions (Wäspi et al., 1998a; Wäspi et al., 1999). Syringolin A, the major variant, was shown not only to induce acquired resistance in rice and wheat after spray application, but also to trigger hypersensitive cell death at infection sites of wheat and Arabidopsis plants infected by compatible powdery mildew fungi (Michel et al., 2006; Wäspi et al., 2001). Recently, syringolin A was shown to be an important virulence factor in the interaction of *Pss* B728a with its host plant *Phaseolus vulgaris* (bean), and its cellular target has been identified. Syringolin A irreversibly inhibits the eukaryotic proteasome by a novel mechanism, representing a new structural class of proteasome inhibitors (Clerc et al., 2009; Groll et al., 2008).

Structure elucidation revealed that syringolin A is a tripeptide derivative consisting of an N-terminal valine followed by the two non-proteinogenic amino acids 3,4-dehydrolysine and 5-methyl-4-amino-2-hexenoic acid, the latter two forming a twelve-membered macrolactam ring. The N-terminal valine is in turn linked to a second valine via an unusual ureido group (Figure 1A) (Wäspi et al., 1998a). The minor variants syringolin B to syringolin F differ from syringolin A by the substitution of one or both valines with isoleucine residues, by the substitution of 3,4-dehydrolysine with lysine, and by combinations thereof (Wäspi et al., 1999). The structure of syringolin A suggested that it was synthesized by a non-ribosomal peptide synthetase (NRPS), large modular enzymes that activate and condense amino acids according to the thiotemplate mechanism (for reviews see e.g. Finking and Marahiel, 2004; Marahiel et al., 1997; von Döhren et al., 1999). We previously cloned and delimited by mutational analysis a genomic region from *Pss* B301D-R containing five open reading frames

(*sylA*-*sylE*) necessary for syringolin biosynthesis (Figure 1B; (Amrein et al., 2004)). Whereas *sylA* and *sylE* encode a putative transcription activator and an exporter, respectively, *sylC* encodes a typical NRPS module predicted to activate valine, whereas *sylD* codes for two additional NRPS modules (of which the first is predicted to activate lysine and the second is predicted to activate valine [10]) and a type I polyketide synthetase (PKS) module. Type I PKS are also modular enzymes that, similar to fatty acid synthesis, extend a starter molecule by condensation/decarboxylation of malonate extender units (for reviews see e.g. Fischbach and Walsh, 2006; Hopwood, 1997). The analysis of the structure and architecture of the *syl* gene cluster led to the postulation of a model that completely accounts for the biosynthesis of the tripeptide part of syringolin A, including its ring structure with the 5-methyl-4-amino-2-hexenoic acid and the 3,4-dehydrolysine (Figure 1C, (Amrein et al., 2004)). However, although the addition of the ureido group and its attached second valine could not be explained by the model, the *syl* gene cluster did not contain additional open reading frames, which, if present, could potentially have been involved in the biosynthesis of this unexplained part.

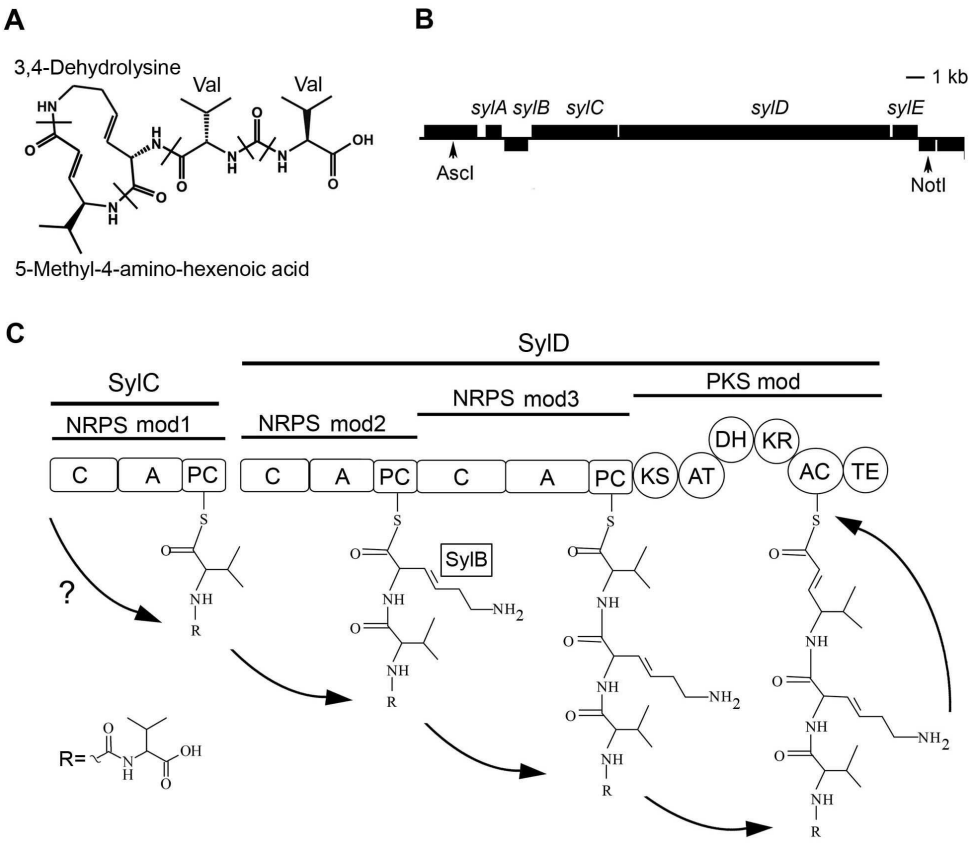


Figure 1. Structure and biosynthesis model of syringolin A.

A. Structure of syringolin A. Amino acid constituents are delimited by bars. Val, valine.

B. Genomic region of *Pss* B301D-R containing the *sylA-sylE* genes. Boxes above and below the line denote ORFs on the top and the bottom strand, respectively. Arrows indicate restriction sites used for cloning of the gene cluster into the cosmid pPL3syl. The *sylA, sylB,* and *sylE* genes encode a LuxR-type transcription activator, a rhizobitoxin desaturase-like protein thought to desaturate the lysine residue, and an efflux transporter, respectively. The *sylC* gene encodes an NRPS module, while *sylD* codes for two NRPS modules and one PKS module.

C. Biosynthesis model of the tripeptide part of syringolin A. The open boxes represent domains in modules of the syringolin A synthetase labeled with C, condensation domain; A, adenylation domain; PC, peptide carrier protein; KS, ketoacyl synthase; AT, acyl transferase; DH, dehydratase; KR, ketoreductase; AC, acyl carrier protein; TE, thioesterase. The A domains of the NRPS modules are thought to activate valine (NRPS mod1), lysine (NRPS mod2), and valine (NRPS mod3). The question mark indicates the unexplained synthesis and attachment of this group. The figures are adapted from Amrein et al. (2004).

Here we show that the genes *sylA-sylE* are sufficient to direct the biosynthesis of *bona fide* syringolin A when heterologously expressed in *Pseudomonas putida* and *Pss* SM, two organisms which do not produce syringolin A and have no *syl* gene cluster

homologue in their genomes. Thus, biosynthesis of the ureido group with its attached terminal valine is achieved without additional syringolin A-specific genes (*i. e.* genes with no other function than in syringolin A biosynthesis). We hypothesized that biosynthesis of the ureido group would most likely be accomplished by the product of the *sylC* gene, which would, in addition to the extracyclic peptidyl valine, also activate the terminal valine and join the two residues by incorporation of a carbonyl group derived from hydrogen carbonate/carbon dioxide, thus forming the ureido moiety. We demonstrate by NMR spectroscopic analysis of syringolin A isolated from *Pss* cultures grown in the presence of $NaH^{13}CO_3$ that the ^{13}C isotope is preferentially found at the position of the ureido carbonyl atom. These results support our hypothesis, which may be of relevance for the hitherto unknown biosynthesis of other ureido-group-containing NRPS products largely known to be produced by cyanobacteria (Gesner-Apter and Carmeli, 2008; Harada et al., 1995; Matthew et al., 2008; Muller et al., 2006; Murakami et al., 1997; Okumura et al., 2009; Schmidt et al., 1997; Williams et al., 1996).

2.3 Results

2.3.1 Biosynthesis of syringolin A in heterologous organisms

In order to test whether the *sylA-sylE* gene cluster was sufficient to direct syringolin A biosynthesis, we constructed a cosmid containing the *sylA-sylE* genes but no other complete gene by taking advantage of *Asc*I and *Not*I restriction sites flanking the *syl* gene cluster (Figure 1B). Southern blot analysis of *Asc*I/*Not*I-digested genomic DNA of *Pss* B301D-R probed with a *sylA* gene fragment labeled the expected 22 kb fragment and thus confirmed the uniqueness of the restriction sites in the relevant genome region (data not shown). Thus, *Pss* B301D-R genomic DNA digested with *Asc*I and *Not*I was separated by agarose gel electrophoresis. Fragments in the 20-23 kb size

range were eluted and cloned into the wide host range cosmid pLAFR3 (Staskawicz et al., 1987). After packaging into lambda phages and transfection into *E. coli* XL-1Blue, the library was screened with a radiolabeled *sylA* gene probe. Positive clones were isolated and confirmed to contain the complete *syl* gene cluster by PCR amplification and sequencing of the expected insert ends. One of the confirmed clones was designated pPL3syl and chosen for further work.

To test the functionality of pPL3syl, the markerless *Pss* B301D-R mutant Δsyl was constructed in which the complete *syl* gene cluster was deleted. The pPL3syl cosmid was then mobilized into the Δsyl deletion mutant by triparental mating. We previously showed that infiltration of syringolin A-producing *Pss* strains or isolated syringolin A into rice leaves leads to the accumulation of transcripts corresponding to the defense-related *Pir7b* gene (encoding an esterase; (Wäspi et al., 1998b)), whereas strains or mutants unable to synthesize syringolin A do not activate this gene (Reimmann et al., 1995; Wäspi et al., 1998a). Syringolin A was originally identified and isolated based on its action on the *Pir7b* gene in rice (Wäspi et al., 1998a). We thus infiltrated the B301D-R wild-type strain, the syringolin-negative mutants Δsyl and sylA_KO (contains a plasmid insertion interrupting the *sylA* transcription activator gene (Amrein et al., 2004)), as well as Δsyl (pPL3syl), the deletion mutant complemented with pPL3syl, into rice leaves. RNA was extracted and subjected to gel blot analysis with regard to *Pir7b* transcript accumulation. As expected and in contrast to the wild type, the syringolin A-negative mutants did not induce *Pir7b* transcript accumulation, whereas the deletion mutant complemented with the pPL3syl cosmid led to a much stronger induction of the *Pir7b* gene (Figure 2A). This strongly suggested that pPL3syl contained a functional *syl* gene cluster able to direct syringolin A synthesis in the Δsyl deletion mutant. This does not exclude the possibility that genes not present in the *syl* gene cluster are necessary for syringolin A production because such genes would also be present in the Δsyl mutant background.

Next we wanted to mobilize pPL3syl into *Pseudomonas* strains not carrying *syl* gene homologs and lacking syringolin A production as evidenced by PCR, DNA gel blot analysis of genomic DNA, high performance liquid chromatography (HPLC) analysis of culture supernatants with regard to syringolin A content, infiltration into rice leaves followed by monitoring of *Pir7b* transcript accumulation, and whole genome sequence comparisons where possible (data not shown). After repeated unsuccessful attempts to transfer pPL3syl into the *P. syringae* pv. *tomato* DC3000 strain (all tetracycline-resistant putative transformants analyzed contained deletion variants of pPL3syl), the cosmid was successfully transferred into the non-pathogenic bacterium *P. putida* P3 (Senior et al., 1976) and *Pss* SM, a strain originally isolated from wheat (Reimmann et al., 1995; Smith and Métraux, 1991). Gel blot analysis of RNA extracted from rice leaves infiltrated with parental and transformed strains showed that, as expected, *P. putida* P3 and *Pss* SM did not induce *Pir7b* transcript accumulation. In contrast, both strains lead to *Pir7b* gene induction when carrying the pPL3syl cosmid (Figure 2B), suggesting that pPL3syl conferred the ability for syringolin A biosynthesis to these strains.

To confirm this, the transformed strains were grown in shaken cultures in SRM_{AF} medium and conditioned media were analyzed by HPLC. As shown in Figure 3, both strains produced a compound eluting at 15.5 min, the elution time of the syringolin A standard. Peaks were collected from multiple HPLC runs and subjected to mass spectrometry. HPLC-high resolution-electrospray ionization-mass spectrometry (HPLC-HR-ESI-MS) of the peaks from *Pss* SM and *P. putida* P3 carrying pPL3syl, and the *Pss* B301D-R wild type showed quasi-molecular ions $[M+H]^+$ at *m/z* 494.29808 (1.5 ppm difference from calculated exact mass), 494.29653 (1.1 ppm), and 494.29799 (1.4ppm), respectively, matching the empirical formula $C_{24}H_{40}N_5O_6^+$ (protonated adduct of syringolin A; calculated exact mass 494.29731). We conclude from these experiments that the *syl* genes contained in pPL3syl are sufficient to direct syringolin A

biosynthesis in these heterologous strains and no further syringolin A-specific genes, *i.e.* genes that exclusively function in syringolin A biosynthesis, are necessary.

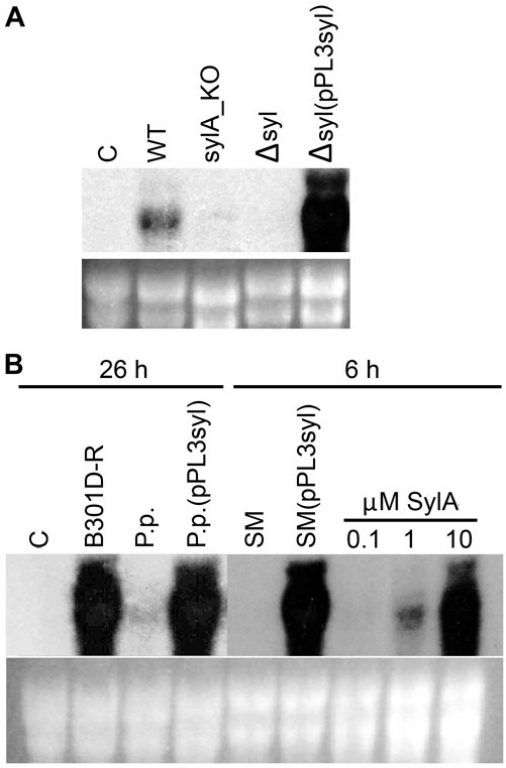


Figure 2. Gel blot analysis of Pir7b transcript accumulation

A. RNA was extracted from rice leaves infiltrated with water (C), *Pss* B301D-R (WT), a *sylA* plasmid insertion mutant (sylA_KO), a *syl* cluster deletion mutant (Δsyl), and Δsyl (pPL3syl), the deletion mutant complemented with the wild-type *syl* gene cluster. Top panel, autoradiogram (exposed for 5 h); bottom panel, ethidium bromide (EtBr)-stained agarose gel.

B. Lanes were loaded with RNA extracted from rice leaves infiltrated as indicated. C, water control; B301D-R, *Pss* wild-type strain; P.p, *P. putida* P3; P.p. (pPL3syl), *P. putida* P3 transformed with the *syl* gene cluster; SM, *Pss* SM; SM (pPL3syl); *Pss* SM transformed with pPL3syl , and syringolin A solutions of the indicated concentrations. Top panel, autoradiogram (exposure times indicated on top), bottom panel, EtBr-stained gel.

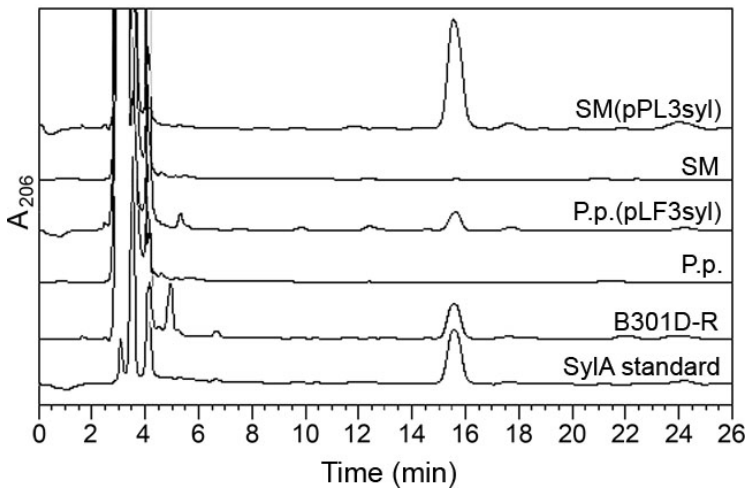


Figure 3. HPLC analysis of syringolin A content in conditioned SRM_{AF} media.

Conditioned media were sterile-filtrated and 20-µl-aliquots were loaded on the column. Absorption was monitored at 206 nm. Labels of HPLC traces are the same as in the legend to Figure 2B.

2.3.2 The ureido carbonyl group of syringolin A is incorporated from bicarbonate/carbon dioxide

The above results raised the question of how the ureido-valine is synthesized and incorporated into syringolin A. We hypothesized that this would most likely be accomplished by the product of the *sylC* gene, which would, in addition to the N-terminal valine of the tripeptide part of syringolin A, also activate the second valine and join the two residues via their amino groups formally by amidation of carbonic acid, thus forming the ureido moiety. If true, feeding syringolin A-producing cultures with ^{13}C-labeled hydrogen carbonate should result in syringolin A that is preferentially labeled with ^{13}C at the ureido carbonyl position. Thus, *Pss* B301D-R transformed with pOEAC, a plasmid carrying the *sylA* transcriptional activator gene under the control of the *lacZ* promoter, was grown in SRM_{AF} medium. After 48 h, ^{13}C-labeled sodium hydrogen carbonate was added to a final concentration of 70 mM and the culture was further grown for 20 h. Syringolin A was isolated from conditioned medium as described (Wäspi et al., 2001) and subjected to ^{13}C NMR analysis.

The spectrum of labeled syringolin A was normalized in order to get the same signal intensities for the valine methyl groups as in the unlabeled sample. Comparison of the normalized NMR spectra revealed that the signal from the ureido carbon atom in ^{13}C-labeled syringolin A was 45-fold stronger than the corresponding signal from unlabeled syringolin A (Figure 4). Inspection of the resolved ^{13}C satellite of the valine methyl group at lowest field in the ^{1}H spectrum of labeled syringolin A (data not shown) suggests a ^{13}C content close to natural abundance. Therefore, the 45-fold signal enhancement in labeled syringolin A directly corresponds to the absolute ^{13}C enrichment at this site. The normalized signal strengths of all other C atoms were equal in labeled and unlabeled syringolin A, with the exception of the C4 position of 3,4-dehydrolysine, whose signal was enhanced approximately 16-fold in ^{13}C-labeled syringolin A (Figure 4A, B). Inspection of biosynthetic pathways using the KEGG database (Kanehisa et al., 2008) revealed that this can be attributed to a carboxylation reaction in the biosynthesis of lysine. The C4 atom of lysine represents the C4 atom of L-aspartate-4-semialdehyde, a derivative of aspartate, which is condensed to pyruvate to yield the intermediary compound L-2,3-dihydrodipicolinate in bacterial lysine biosynthesis. The C4 atom of aspartate in turn originates from the carboxylation of pyruvate to oxaloacetate, an intermediary compound in the tricarboxylic acid cycle, which is transaminated to aspartate. Thus, enhanced ^{13}C-labeling of lysine with $H^{13}CO_3^-$ at the C4 position is to be expected. We note that malonate will also be labeled by $H^{13}CO_3^-$ as it is derived from acetate by carboxylation. However, the label will be removed by the condensation/decarboxylation of malonate to the peptide chain during syringolin A biosynthesis. We conclude from this analysis that our hypothesis is correct, *i.e*. that the ureido carbonyl moiety in syringolin A originates from the incorporation of hydrogen carbonate/carbon dioxide.

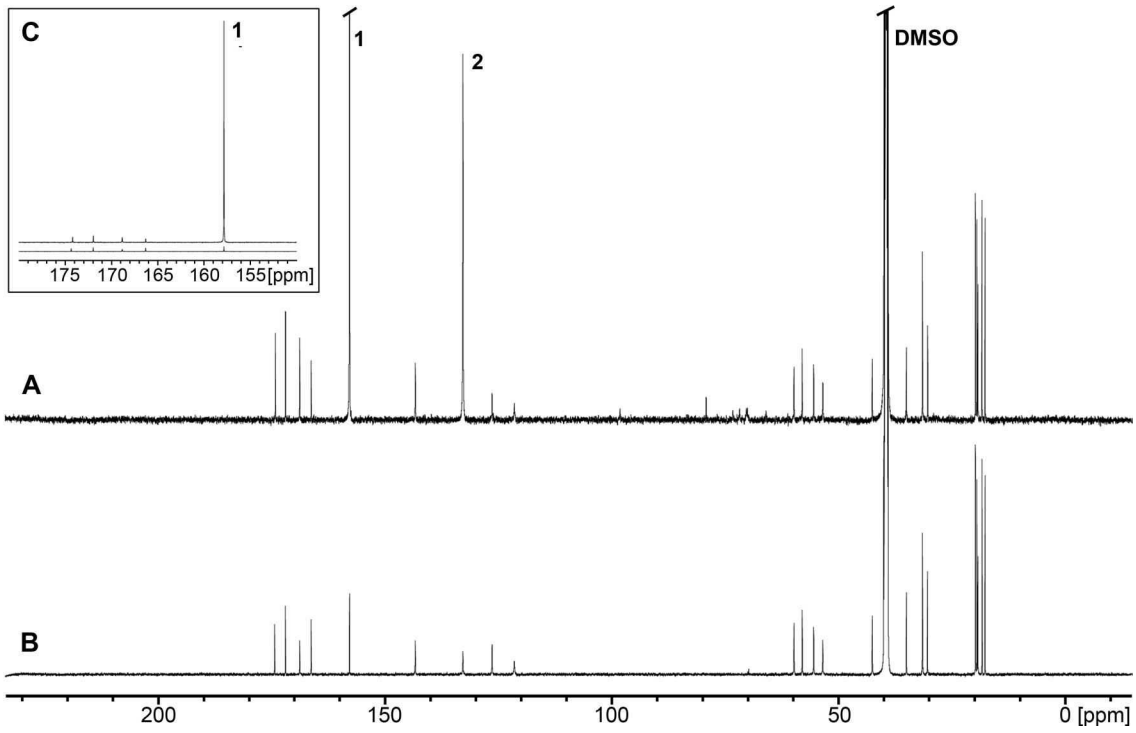


Figure 4. ^{13}C-NMR spectra of *in vivo* NaH$^{13}CO_3$-labeled and unlabeled syringolin A.

The spectra of NaH$^{13}CO_3$-labeled **(A)** and unlabeled **(B)** syringolin A have been scaled to give equal signal intensities for the methyl groups of the valine residues (17.4, 17.5, 19.0, and 19.1 ppm shifts). The signals at 157.8 ppm (marked 1; clipped off) and 132.8 ppm (marked 2) correspond to the ureido carbonyl group and the lysine C4 position, respectively. DMSO, DMSO solvent signal.

C. Scaled-down version of part of the spectra given in (A) and (B) to show the difference in signal intensity of the ureido carbonyl group in NaH$^{13}CO_3$-labeled and unlabeled syringolin A, respectively.

2.4 Discussion

We have demonstrated that the *syl* gene cluster is sufficient to direct syringolin A synthesis in heterologous organisms. Although the biosynthesis model presented earlier (Amrein et al., 2004) plausibly explained every structural feature of the syringolin A tripeptide part through the enzymatic actions of the *sylB, sylC,* and *sylD* gene

products, the generation and condensation of the ureido valine remained enigmatic. As the other genes present in the *syl* cluster encode a transcriptional activator (*sylA* gene) and an exporter (*sylE* gene), a plausible hypothesis was that the *sylC*-encoded NRPS module not only activated the N-terminal peptidyl valine, but also the ureido valine, and that the ureido carbonyl moiety is incorporated from hydrogen carbonate/carbon dioxide. As shown above, *in vivo* labeling of syringolin A with ^{13}C-hydrogen carbonate supports this hypothesis. Currently, we can only speculate how this is achieved. One possibility is that the quaternary syringolin A synthetase complex may contain two (not necessarily identical) molecules derived from the *sylC* gene per SylD polypeptide. Both *sylC* gene products would activate valine, or, to a certain degree, isoleucine in minor syringolin variants (Wäspi et al., 1999). The first valine would then be carbamylated by HCO_3^-/CO_2, perhaps without the action of another enzyme, as has been reported for the carbamylation of a catalytic lysine residue in β-lactamases of class D (Golemi et al., 2001; Maveyraud et al., 2000). The ureido moiety would then be formed by amide bond formation between the carbamylated valine and the second valine. In this scenario, it remains unclear how the first valine, which, like the second one, is envisioned to be bound to the peptide carrier protein domain by a thioester bond, is released upon ureido bond formation. It is also conceivable that ureido bond formation is achieved by a single SylC protein, which contains a condensation domain usually absent from starter modules that may be involved. To clarify these issues, more structural information about the large syringolin A synthetase and the SylC module must be obtained. The reconstitution of the enzymatic activities of the module(s) derived from the *sylC* gene *in vitro* will be challenging.

In addition to the syringolin family of compounds, a number of other natural cyclic peptides mostly isolated from cyanobacteria have been described in the literature that contain extracyclic ureido groups linking two different amino acids. These include anabaenopeptins from *Anabaena, Oscillatoria*, and *Planktothrix* species (Gesner-Apter

and Carmeli, 2008; Harada et al., 1995; Murakami et al., 1997; Okumura et al., 2009), ferintoic acids from *Mycrocystis aeruginosa* (Williams et al., 1996), pompanopeptins from *Lyngbya confervoides* (Matthew et al., 2008), as well as mozamides and brunsvicamides, compounds of presumably cyanobacterial origin isolated from sponges (Muller et al., 2006; Schmidt et al., 1997). Bicarbonate/CO_2 may also be the source of the ureido carbonyl group joining two extracyclic amino acids in the biosynthesis of these compounds, which, to our knowledge, has not been elucidated so far.

Conclusions

Our results show that the *syl* biosynthesis gene cluster was sufficient to direct the biosynthesis of *bona fide* syringolin A, including the enigmatic ureido group joining two amino acids. They reveal the source of the ureido carbonyl group to be bicarbonate/carbon dioxide, which we hypothesize is incorporated by carbamylation of valine mediated by the *sylC* gene product(s). A similar mechanism may also play a role in the biosynthesis of other ureido-group-containing NRPS products known largely from cyanobacteria.

2.6 Methods

Construction and expression of pPL3syl

Unless stated otherwise, standard protocols were used (Ausubel et al., 1987). Genomic DNA from *Pss* B301D-R was isolated and 11 µg were digested with the restriction enzymes *Asc*I and *Not*I. *Asc*I and *Not*I sites are both unique in the *syl* gene region (GenBank: AJ548826) located at position 2052 and 24124, respectively, within the ORFs flanking the *sylA-sylE* ORFs (3507-23596). A DNA gel blot was prepared with 1

µg of the digested DNA and probed with a ^{32}P-labeled *sylA* gene fragment PCR-amplified from genomic DNA with primers P1 (5'-ccatcgatggagtagagtgatggc) and P2 (5'-ggaattcttacaaaattcccatcttg). The rest of the digested DNA was separated on a 0.4% agarose gel and the DNA in the 20-23 kb size range was cut out, electrophoretically eluted into a dialysis bag (10 kDa cutoff), extracted first with 1 volume of phenol and then with 1 volume of phenol-chloroform-isoamylalcohol (25:24:1), precipitated with ethanol and finally taken up in TE (10 mM Tris-HCl, pH 8; 1 mM EDTA). Fragments were ligated into the *Hin*dIII/*Bam*HI-cut broad host range cosmid vector pLAFR3 (Staskawicz et al., 1987) using adaptors prepared by annealing the oligonucleotide 5'-cgcgccaagcttcca with 5'-agcttggaaagcttgg (*Asc*I/*Hin*dIII adaptor) and 5'-ggccgctagtcaggag with 5'-gatcctcctgactagc (*Not*I/*Bam*HI adaptor), respectively. Ligation products were packaged into lambda phage particles using the Gigapack III Gold Packaging Kit (Stratagene, La Jolla, California) and the library was plated out on *E. coli* XL1-Blue (Stratagene) and screened according to the instructions of the manufacturer using the ^{32}P-labeled *sylA* gene fragment described above as a probe. Positive clones were isolated and confirmed to contain the complete *syl* gene cluster by PCR amplification and sequencing of the insertend fragments using primers 5'-ccggcctacacgcattc (*sylA* end) and 5'-agcaacctggatgtacgg (*sylE* end) with the respective adaptor oligonucleotides (see above).

pPL3syl was transferred from XL1-Blue to *Pseudomonas* strains by triparental mating using the *E.coli* helper strain HB101 (pRK600) (Christensen et al., 1999; Finan et al., 1986).

Construction of the *syl* gene cluster deletion mutant Δsyl

Two fragments of 783 bp and 655 bp length flanking the *syl* gene cluster on the 5' and 3' side, respectively, were amplified by PCR from *Pss* B301D-R genomic DNA using the primer pairs P3 (5'-cgggatcc**a**acctgaaatgggagagtc; base given in bold at position

2297 in GenBank:AJ548826) and P4 (5'-agcgcgaggac**c**tcaatgtgaaaacaacg; bold base at position 3072), and P5 (5'-tcacattgag**t**cctcgcgctggtaacc; bold base at position 23600) and P6 (5'-**t**tctgcagtcaagcctgacgaaaagc; bold base at position 24247), respectively. The two bands were isolated and joined by overlap extension PCR using primers P3 and P6 to yield a fragment flanked by *Bam*HI and *Pst*I restriction sites in which the *syl* gene cluster from position 3073-23599 (GenBank:AJ548826) was missing. The deletion is nearly identical with the one in the completely sequenced *P. syringae* pv. *tomato* DC3000 (GenBank:NC_004578.1), which does not contain a *syl* gene cluster. The fragment was cut with *Bam*HI and *Pst*I and cloned into the respective restriction sites in the cloning box of the suicide vector pME3087 (Tc^R, ColE1 replicon (Voisard et al., 1994)). The recombinant plasmid was transformed into *E. coli* S17-1 (*thi pro hsdR recA*; chromosomal RP4 (Tra^+ Tc^S Km^S Ap^S; transfer gene-positive, tetracycline-sensitive, kanamycin-sensitive, ampicillin-sensitive) (Simon et al., 1983)) and mobilized into *Pss* B301D-R. Tetracycline-resistant colonies were grown in LB medium over night at 28° C on a rotary shaker (220 rpm). For selection of tetracycline-sensitive colonies, the overnight cultures were diluted 100-fold with LB. After 2 h of growth, tetracycline was added (20 µg/ml final concentration) and the cultures were grown for 1 h, after which the bactericide carbenicillin (2 mg/ml final concentration) was added for 3 h. The bacteria were then collected by centrifugation, and after washing them twice in LB, the selection procedure was repeated another 3 times. The cultures were then replica-plated on LB plates with and without tetracycline (10 µg/ml) and tetracycline-sensitive colonies were isolated (about 2-3 %). The desired deletion mutants were distinguished from wild-type revertants and verified by sequencing of a 1.7 kb DNA fragment amplified from genomic DNA by PCR using primers 5'-attactcgaccagttccg and 5'-ttacgcaatggtatgatgc which are located outside the fragment cloned into the suicide vector pME3087 at position 2113 and 24385 (GenBank:NC_007005.1), respectively.

Construction of pOEAC

The *sylA* ORF was amplified from genomic DNA using the primers P7 (5'-cc*atcgat*ggagtagagtg**atg**gc; *Cla*I site in italics, translation initiation codon indicated in bold) and P8 (5'-g*gaattc***tta**caaaattcccatcttg; reverse primer; *Eco*RI site in italics, reverse stop codon in bold), digested with *Cla*I and *Eco*RI, and cloned into the respective polylinker sites of the pME6001 (Gm^R) expression vector (Blumer et al., 1999), thereby placing it under the control of the *lacZ* promoter. The resulting plasmid was named pOEA. As it turned out that pOEA did not confer gentamycin resistance in SRM_{AF} medium, pOEAC was used, a derivative of pME6014 (Tet^R) (Schnider-Keel et al., 2000), which, in addition to the *lacZ::sylA* chimeric gene, contained a *sylC::lacZ* reporter fusion gene in opposite orientation (the reporter gene is of no relevance in the present context). To construct pOEAC, the *lacZ::sylA* fusion gene was amplified from pOEA with primers P8 (see above) and P9 (5'-accgtccaac**a**ttaatgcagctgg; upstream of *lac* promoter; bold base complementary to position 987 of pBluescript vector (GenBank:X52329)) and joined with a *sylC* promoter fragment (position 5409-5649 of GenBank:AJ548826) that was amplified with primers P10 (5'-ctgcattaat**g**ttggacggtctgc; bold base at position 5409) and P11 (5'-aa*ctgcag***t**catgacggcctcggat; *Pst*I site in italics, bold base at position 5649) by overlap extension PCR using primers P8 and P11. The resulting fragment was digested with *Eco*RI and *Pst*I and cloned between the respective sites in the polylinker of pME6014.

Bacterial infiltration of rice leaves and RNA gel blot analysis

Bacterial strains were grown on a rotary shaker (220 rpm) over night at 28° C in LB containing, where appropriate, 10 µg/ml tetracycline. Bacteria were pelleted by centrifugation, washed twice in distilled water, resuspended in distilled water at an optical density at 600 nm (OD_{600}) of 0.4 (approximately 10^8 cfu), and infiltrated into first leaves of 14-day-old rice plants (*Oryza sativa* cv. Loto; supplied by Terreni alla Maggia,

Ascona, Switzerland) as described previously (Reimmann et al., 1995). RNA was extracted from infiltrated leaves 16 h after infiltration and subjected to gel blot analysis using a ^{32}P-labeled *Pir7b* cDNA probe (GenBank:Z34270 (Reimmann et al., 1995)) according to standard procedures (Ausubel et al., 1987).

HPLC analysis and mass spectrometry of syringolin A

To analyze conditioned media with regard to syringolin A content, *Pseudomonas* strains were grown in SRM_{AF} medium (Gross, 1985; Mo and Gross, 1991b) at 28°C for 60 h on a rotary shaker (220 rpm). Bacteria were pelleted by centrifugation and the supernatant was sterile filtered (0.22 µm pore size). Two-hundred-microliter aliquots were acidified with trifluoroacetic acid (TFA; 0.3% final concentration) and subjected to reverse-phase HPLC with a Reprosil 100-5 C_{18} 250/4.6 column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) on a Dionex UltiMate 3000 system (Dionex Corporation, Sunnyvale, CA). Elution was performed isocratically with 20% acetonitrile and 0.06% TFA in water at a flow rate of 1 ml/min.

High-resolution electrospray mass spectra were recorded on a Bruker maXis QTOF-MS instrument (Bruker Daltonics GmbH, Bremen, Germany). The samples were dissolved in MeOH and analyzed via continuous flow injection at 3 µl/min. The mass spectrometer was operated in positive ion mode with a capillary voltage of 4 kV, an endplate offset of –500 V, nebulizer pressure of 5.8 psig, and a drying gas flow rate of 4 l/min at 180° C. The instrument was calibrated with a Fluka electrospray calibration solution (Sigma-Aldrich, Buchs, Switzerland) that was 100 times diluted with acetonitrile. The resolution was optimized at 30’000 FWHM in the active focus mode. The accuracy was better than 2 ppm in a mass range between *m/z* 118 and 2721. All solvents used were purchased in best LC-MS qualities.

^{13}C-labeling and NMR Spectroscopy

Pss B301D-R was transformed with pOEAC, and grown in LB containing 10 µg/ml tetracycline on a shaker at 28° C until an OD_{600} of approximately 0.5 was reached. Bacteria were collected by centrifugation, washed twice with SRM_{AF} medium, and taken up in SRM_{AF} medium at an OD_{600} of 0.3. Fifty-ml cultures were inoculated with 0.01 volume of the bacterial suspension and incubated at 28° C on a shaker (220 rpm). After 48 h, $NaH^{13}CO_3$ (98%; Sigma-Aldrich, Buchs, Switzerland) was added to a final concentration of 70 mM and incubation was continued for 20 h. Bacteria were pelleted and syringolin A was isolated from sterile-filtrated conditioned media as described (Wäspi et al., 2001).

^{1}H broadband decoupled ^{13}C NMR spectra were recorded at 25 °C on a Bruker Avance III 600 MHz spectrometer equipped with a cryogenic 5 mm CPDCH probe head optimized for ^{13}C detection. Two samples were prepared by dissolving 200 µg of labeled syringolin A in 130 µl DMSO-d6 and 5 mg of unlabeled syringolin A in 750 µl DMSO-d6, respectively. The labeled sample was transferred to a 3 mm Shigemi tube, the unlabeled sample was transferred to a regular 5 mm NMR tube. The spectral width in both spectra was 248.5 ppm, the transmitter was set to 100 ppm. The excitation pulse angle was set to 45°. The acquisition time was 2.1 s with a waiting time of 0.3 s between two scans. Both spectra were ^{1}H broadband decoupled using the waltz16 composite-pulse decoupling scheme. The resulting fid consisted of 157890 total data points. For the unlabeled syringolin A sample 4000 scans were accumulated. For the labeled syringolin A sample 29605 scans were accumulated. Both spectra were zero filled to 131072 complex data points and processed using an exponential line broadening of 2 Hz. The samples contained no internal chemical shift reference and the spectra were referenced to the solvent peak (39.5 ppm). By comparison with chemical shifts listed in (Wäspi et al., 1998a) the signals at 157.8 ppm and 132.8 ppm

were assigned to the ureido CO group and the olefinic C at position 4 in the 3,4-dehydrolysine moiety, respectively.

Authors' contributions

CR carried out the majority of experiments. The pPL3syl cosmid and the Δsyl deletion mutant were constructed by MT and MM, respectively. Mass spectrometry and NMR spectroscopy were performed and analyzed by LB and MOE, respectively. BS performed RNA gel blot analyses in the rice infiltration experiments. RD, CR, and BS designed experiments and RD wrote a draft manuscript. All authors provided critical inputs to the manuscript.

Acknowledgements

We thank Zsuzsa Hasenkamp for expert technical assistance, and Enrico Martinoia, Stefan Hörtensteiner, Markus Kaiser, and André Bachmann for discussions and helpful comments on the manuscript. Financial support by the Swiss National Science Foundation (grant 3100A0-115970 to RD) is acknowledged.

3.

REGULATION OF BIOSYNTHESIS OF SYRINGOLIN A, A *PSEUDOMONAS SYRINGAE* VIRULENCE FACTOR TARGETING THE HOST PROTEASOME

Christina Ramel [1], Nando Baechler [1], Michel Hildbrand [1], Martin Meyer [1], David Schädeli [1], and Robert Dudler [1 §]

MPMI

Submitted March 2012

Reviewed and recommended for publication

April 2012

[1] Institute of Plant Biology, University of Zurich, Zollikerstrasse 107, CH-8008 Zurich, Switzerland

[§] Address for correspondence: Robert Dudler, rdudler@botinst.uzh.ch

3.1 Abstract

Many strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* synthesize the virulence factor syringolin A, which irreversibly inactivates the eukaryotic proteasome. Syringolin A, a peptide derivative, is synthesized by a mixed non-ribosomal peptide/polyketide synthetase encoded by five clustered genes, *sylA-sylE.* Biosynthesis of syringolin A, previously shown to be dependent on the GacS/GacA two component system, occurs *in planta,* and *in vitro*, but only under still culture conditions in a defined medium. Here we show that the *sylC, sylD, and sylE* genes of *Pss* B301D-R form an operon transcribed by promoter sequences located between the *sylCDE* operon and the *sylB* gene residing on opposite strands. Assays of overlapping *sylB* and *sylCDE* promoter deletions translationally fused to the *lacZ* gene defined promoter sequences required for gene activity both *in vitro* and *in planta*. Activation of both promoters depended on the *sylA* gene encoding an HTH LuxR-type transcription factor which was shown to directly bind to the promoters. Activity of the *sylA* gene in turn required a functional *salA* gene, which also encodes an HTH LuxR-type transcription factor. Furthermore, evidence is presented that acyl-homoserine lactone-mediated quorum sensing regulation is not involved in syringolin A biosynthesis, but that oxygen concentration appears to play a role.

3.2 Introduction

Many strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* (*Pss*) secrete syringolins, a family of closely related cyclic peptide derivatives, *in planta* and under suitable *in vitro* culture conditions (Wäspi et al., 1998a; Wäspi et al., 1999). Spray application of syringolin A, the predominant variant, onto powdery mildew-infected wheat and Arabidopsis results in large transcriptome changes and programmed cell death at infection sites (Michel et al., 2006; Wäspi et al., 2001). Syringolin A was also shown to be an important virulence factor in the interaction of *Pss* B728a with bean (*Phaseolus vulgaris*) (Groll et al., 2008). Elucidation of syringolin A's mode of action revealed that it covalently binds to the active site residue of all three catalytic subunits of the eukaryotic proteasome by a novel mechanism, thereby irreversibly inhibiting proteasomal protein degradation (Groll et al., 2008). Recently, experiments with bean and *Arabidopsis thaliana* revealed that proteasome inhibition by syringolin A suppresses the stomatal immune reaction and the salicylic acid-dependent host defense pathway (Schellenberg et al., 2010).

Syringolin A (Figure 1A) is a tripeptide derivative that consists of an N-terminal valine followed by the two non-proteinogenic amino acids 3,4-dehydrolysine and 5-methyl-4-amino-2-hexenoic acid, the latter two forming a 12-membered macrolactam ring structure. The N-terminal valine is linked by a urea moiety to the amino group of a second valine (Wäspi et al., 1998a). In minor variants, one or both valines can be substituted by isoleucine residues, and the 3,4-dehydrolysine by lysine (Wäspi et al., 1999). Syringolins are synthesized by a mixed non-ribosomal peptide synthetase/polyketide synthase (NRPS/PKS) which is encoded by a gene cluster comprising five open reading frames (ORFs) termed *sylA-sylE* (Amrein et al., 2004). These genes are sufficient to direct syringolin A biosynthesis when transferred to *Pseudomonas* strains lacking syringolin A biosynthesis genes (Ramel et al., 2009).

The architecture of these genes and experimental evidence allowed to postulate a biosynthesis model completely explaining syringolin A's structure (Amrein et al., 2004; Imker et al., 2009; Ramel et al., 2009). While *sylC* and *sylD* encode NRPS and PKS modules of the syringolin A synthetase, the *sylB* gene is thought to be responsible for the desaturation of the 3,4-dehydrolysine residue (Amrein et al., 2004). The *sylA* gene encodes a helix-turn-helix (HTH) LuxR-type DNA-binding domain protein hypothesized to be a transcriptional regulator, whereas the *sylE* gene codes for a major facilitator superfamily protein postulated to represent the syringolin A exporter (Amrein et al., 2004).

The regulation of syringolin A biosynthesis appears to be complex. Syringolin A production was shown to be dependent on the *lemA* gene (Reimmann et al., 1995; Wäspi et al., 1998a) encoding the sensor part of the GacS/GacA two-component system, which controls many aspects of pathogenicity such as e.g. phytotoxin and protease production, and lesion formation (Hrabak and Willis, 1992; Rich et al., 1992; Rich et al., 1994). Syringolin A is produced *in planta*, even if bacteria are infiltrated into non-host species, and *in vitro* under still culture conditions (Wäspi et al., 1998a) in a medium originally optimized for the production of the phytotoxin syringomycin (Gross, 1985; Mo and Gross, 1991b; Mo and Gross, 1991a).

In order to elucidate the regulation of syringolin A biosynthesis, we demonstrate here that the *sylC, sylD,*and *sylE* genes are transcribed as an operon and that the intergenic region between the *sylB* and *sylCDE* genes, which reside on opposite DNA strands, contains the promoters responsible for transcription of these genes. We show that the product of the *sylA* gene directly activates the *sylB* and *sylCDE* genes, and that the *salA* gene, which also encodes a HTH LuxR-type DNA-binding domain protein (Kitten et al., 1998), is required for the activation of *sylA.* In addition, we present evidence that the homoserine lactone quorum sensing system is not involved in syringolin A biosynthesis, but that oxygen concentration appears to play a role.

3.3 Results

3.3.1 The *sylC*, *sylD* and *sylE* genes form an operon

The syringolin A gene cluster consists of the five open reading frames (ORFs) *sylA* to *sylE* (Amrein et al., 2004). The arrangement of the *sylC*, *sylD*, and *sylE* genes suggests that they may be transcribed as an operon, likely regulated together by a promoter residing in the intergenic region between the *sylB* and *sylC* genes (Figure 1B).

Amplification of the intergenic regions between *sylC*, *sylD* and *sylE* from RNA by RT-PCR indicated that these genes are indeed transcribed as a single polycistronic mRNA (Figure 1C). This finding was corroborated by RNA gel blot analysis with RNA extracted from the wild-type strain carrying the *sylA* overexpression construct pOEAC (Ramel et al., 2009) using *sylC* and *sylE* fragments as hybridization probes. As shown in Figure 1D, both the *sylC* and the *sylE* gene fragments labeled a band corresponding to a large RNA of a size consistent with the 18 kb expected for a transcript encompassing the *sylC, sylD*, and *sylE* ORFs, which have a size of 3984, 12570, and 1233 nucleotides, respectively. This result confirmed that these ORFs are transcribed as an operon.

To identify promoter sequences expected to reside within the intergenic region between the *sylB* and *sylC* ORFs, reporter genes were constructed in which the 221 bp intergenic sequence plus 18 bp and 5 bp of the coding regions of the *sylB* and *sylC* gene, respectively, was fused in frame with the *lacZ* ORF of pME6014 in both orientations (Schnider-Keel et al., 2000). These constructs were named psylBp-lacZ and psylCp-lacZ, respectively. We note that in psylCp-lacZ, not the first ATG codon, but an in-frame ATG codon encoding the seventh amino acid of the *sylC* ORF as originally annotated by Amrein et al. (2004) represents the translational initiation codon of the *sylC* gene (see below). Translational *lacZ* fusions after the first ATG were completely inactive.

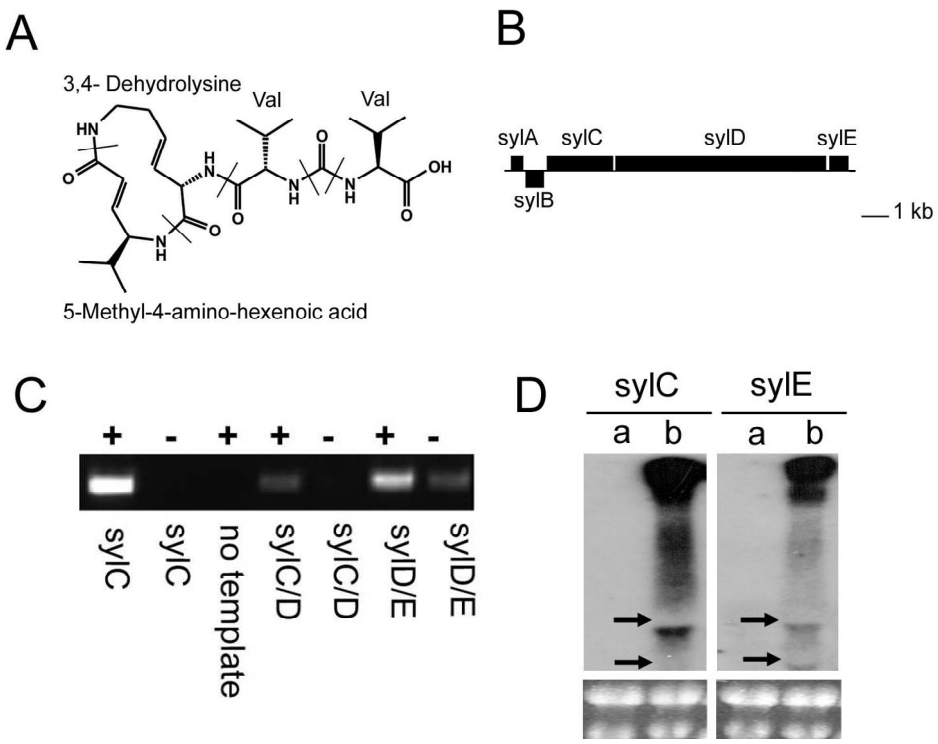


Figure 1. *SylC. sylD, and sylE* are transcribed as an operon.

A, Structure of syringolin A. Amino acid constituents are delimited by bars. Val, valine.

B, Genomic region of *Pss* B301D-R containing the *sylA-sylE* genes (accession no. AJ548826). Boxes above and below the line denote ORFs on the top and the bottom strand, respectively.

C, RT-PCR with primers amplifying a *sylC* gene fragment or the *sylC*/*D* and *sylD*/*E* intergenic regions, respectively. Reactions did (+) or did not (-) contain reverse transcriptase.

D, Top panels: Autoradiogram of an RNA gel blot hybridized with a *sylC* and a *sylE* probe, respectively. RNA was extracted from *Pss* B301D-R from which the complete *syl* gene cluster has been deleted (lane a), or from *Pss* B301D-R overexpressing the *sylA* gene (lane b). Arrows indicate the position of the 23S and 16S rRNA. Bottom panels: EtBr stained gel.

3.3.2 The LuxR-type transcription factor SylA is a positive regulator of the *sylB* and *sylC* promoters

The *sylA* gene encodes a putative protein with a HTH LuxR-type DNA-binding domain. The location of the *sylA* gene and the fact that syringolin A production was abolished if the *sylA* gene was disrupted by plasmid insertion (Amrein et al., 2004) led to the hypothesis that the *sylA* gene product may be a positive regulator of the *sylB* and

sylCDE transcriptional units. To verify this hypothesis, a marker-less *sylA* deletion mutant (ΔsylA) was constructed and transformed with the reporter constructs. In this mutant, the activities of the *sylB* and the *sylC* promoters were strongly decreased as compared to the WT (Table 1).

Table 1. Effect of *sylA* on *sylB* and *sylC* reporter gene activity

Strain[a]	Reporter	B-gal activity[b]	Comment
WT (psylBp-lacZ)	sylB	705 ± 37	
ΔsylA (psylBp-lacZ)	sylB	96 ± 20	
WT (psylCp-lacZ)	sylC	1194 ± 108	
ΔsylA (psylCp-lacZ)	sylC	106 ± 6	
WT (pOEAB)	sylB	6960 ± 149	*sylA* overexpression
ΔsylA (pOEAB)	sylB	7128 ± 106	*sylA* overexpression
WT (pOEAC)	sylC	7923 ± 249	*sylA* overexpression
ΔsylA (pOEAC)	sylC	9600 ± 415	*sylA* overexpression

[a] WT = wild type
[b] β-Galactosidase (β-Gal) activity. ± standard error (n > 15) in Miller units (MU).

Next we placed the *sylA* gene under the control of the *lacZ* promoter onto the *sylB* and *sylC* reporter plasmids, resulting in the *sylA* overexpression constructs pOEAB and pOEAC, respectively. β-galactosidase (β-gal) assays of the wild type and the ΔsylA mutant transformed with these constructs revealed a large increase of the *sylB* and *sylC* promoter activities as compared to the reporter plasmids psylBp-lacZ and psylCp-lacZ in the wild-type background (Table 1). In addition, syringolin A concentrations of conditioned media from the former strains also increased with the same order of magnitude as compared to the wild-type strain carrying the empty vector (data not shown). We conclude from these experiments that SylA is a positive regulator of syringolin A biosynthesis that directly or indirectly activates the *sylB* and *sylC* promoters.

3.3.3 Delimitation of essential *sylB* and *sylC* promoter regions

To delimit sequences necessary for activity of the *sylB* and *sylC* promoters, overlapping deletions of the *sylB-sylC* intergenic DNA fragment were prepared by PCR amplification (Figure 2; see Suppl. Table 2 for primer sequences) that were translationally fused to the *lacZ* reporter gene in pME6014.

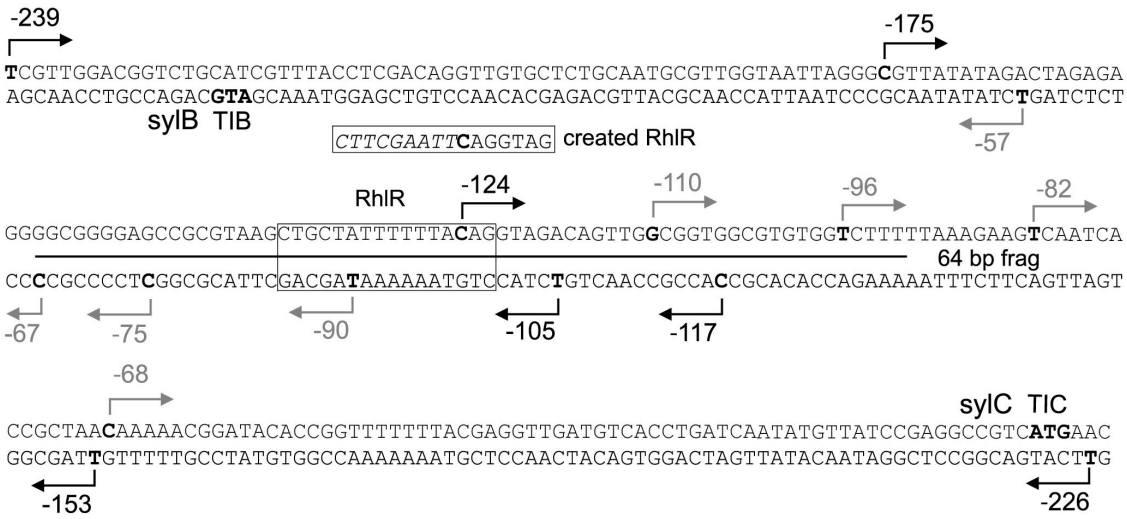


Figure 2. Intergenic region between the *sylB* and *sylC* genes.

Translation initiation codons of the *sylB* (TIB, bottom strand) and *sylC* (TIC, top strand) genes are shown in bold letters. Positions of promoter deletion breakpoints relative to the respective translational initiation codon are indicated with negative numbers above (*sylC*) or below (*sylB*) arrows. Fragments with background promoter activities are indicated with gray arrows, fragments with activities above background with black ones. A 64 bp long subfragment used in MDAE experiments is marked with a bar between the DNA strands. The RhlR-like binding site residing within the 64 bp fragment as well as the one recreated by cloning the -124 bp fragment into the *EcoR*I cloning site (italics) are boxed.

Results of β-gal assays with *Pss* B301D-R strains transformed with these reporter plasmids and grown under still culture conditions are shown in Figure 3.

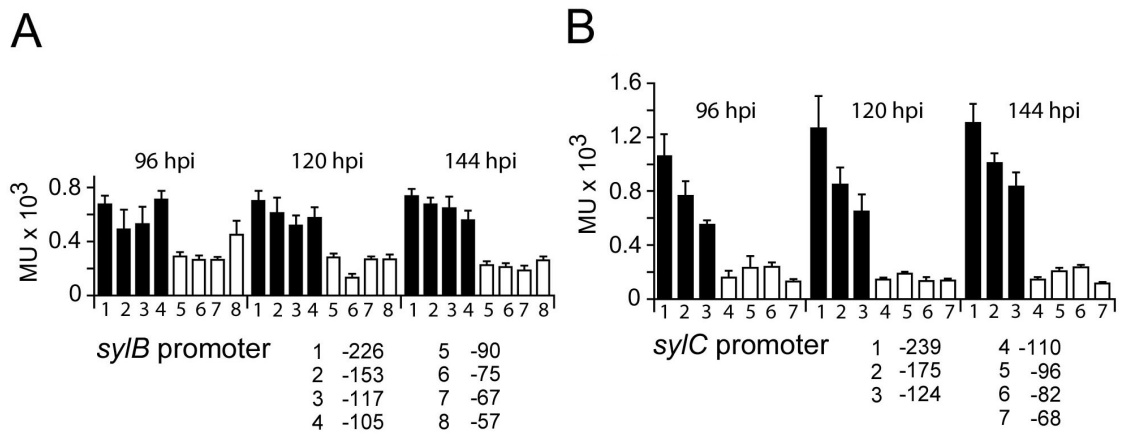


Figure 3. Promoter activities of *sylB* (A) and *sylC* (B) deletion fragments.

Open bars indicate fragments resulting in background activity, black bars fragments with activities above background. Means of data points measured at three representative culture time points (96, 120 and 144 h after inoculation), each in 6 to 11 replicates, are given in Miller units. Error bars indicate the standard error of the mean. Promoter fragments of the *sylB* and the *sylC* genes indicated for the 96 h time point apply analogously to the other time points.

For the *sylB* gene, fragments comprising 226 bp (full length), 153 bp,117 bp, and 105 bp upstream of the translation initiation were active to about the same extent (black colums in Figure 3A), whereas fragments of 90 bp, 75 bp, 67 bp, and 57 bp only led to basal activities (open columns). For the *sylC* gene, promoter fragments encompassing 239 bp (full length), 175 bp, and 124 bp of upstream sequence were active above basal levels (back columns, Figure 3B), although activities seemed consistently to decrease with shorter fragment length. Fragments of 110 bp, 96 bp, 82 bp, and 68 bp resulted in basal activities (open columns). The shortest active *sylB* and *sylC* promoter fragments (105 bp and 124 bp, respectively) overlap for 8 bp at their 5' ends (Figure 2).

To complement these results from *in vitro* cultures, β-gal activity was also monitored *in planta* after infiltration of bacteria harbouring reporter plasmids into leaves of *Nicotiana benthamiana* using a colorigenic substrate (adapted from Hull et al., 1996; Teeri et al., 1989). The results were consistent with the ones obtained from *in vitro*

cultures and identified the 105 bp *sylB* and 124 bp *sylC* promoter fragments as the minimal fragments necessary for promoter activity *in planta* (Figure 4A).

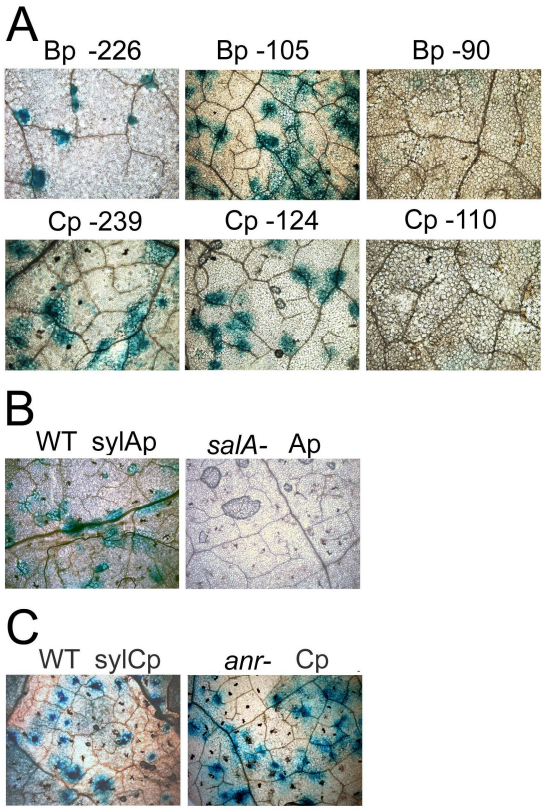


Figure 4. Qualitative β-gal activity staining of bacteria carrying promoter-*lacZ* fusions in leaves of *Nicotiana benthamiana.*

Leaves infiltrated with 10^5 CFU/ml *Pss* B301D-R carrying the indicated promoter-reporter constructs. Leaves were stained with X-Gal after 72 to 96 h.

A, Leaves with bacteria carrying *sylB* (Bp) and *sylC* (Cp) promoter deletion reporter constructs, respectively, were stained for equal amounts of time.

B, *N. benthamiana* leaves infiltrated with wild-type (left panel) and *salA*-mutant bacteria (right panel) harbouring the the psylAp-lacZ reporter plasmid.

C, *N. benthamiana* leaves infiltrated with wild-type (WT) and Psyl3425-orthologue (*anr* homologue)-negative bacteria carrying a full-length *sylC::lacZ* reporter construct stained for the same amount of time.

Analysis of the 221 bp intergenic region with the Virtual Footprint program (http://prodoric.tu-bs.de/vfp/) (Munch et al., 2005) predicted putative imperfect dyad binding sites on both strands with scores (9.6 and 9.79) for the *P. aeruginosa* HTH

LuxR-type response regulator RhlR near the ends of the shortest active *sylB* and *sylC* promoter fragments (boxed in Figure 2). This putative binding site is present in all active, but absent or truncated in all *sylB* promoter fragments exhibiting basal activities. This holds true also for the *sylC* promoter, except for the shortest active deletion (124 bp of upstream sequence), where it is severely truncated (Figure 2). Intriguingly, analysis with the Virtual Footprint program of this deletion clone, including the neighbouring vector sequences, revealed that by chance, a good RhlR binding site with a score of 10.68 was recreated around the *Eco*RI cloning site into which this deletion fragment was cloned (Figure 2), thus suggesting that this putative binding site may also be important for *sylC* transcriptional activation.

3.3.4 An MBP-SylA fusion protein binds to *sylB*/*sylC* promoter fragments

In order to determine whether the SylA positive regulator binds directly to the *sylB* and *sylC* promoter fragments, maltose-binding protein (MBP)-SylA fusion proteins were generated. The *sylA* gene was amplified by PCR, cloned in frame with MBP into the vectors pMal-c2X (for expression in *E. coli*) and pMEKm12 (a derivative of pMal-c2X) for expression in *P. syringae* (Lu et al., 2002b) to yield pMalSylA and pMEKmSylA, respectively. The recombinant plasmids were transformed into *E. coli* DH10B and *Pss* B301D-R.

MBP-tagged proteins were purified from extracts of both organisms by maltose affinity chromatography and subjected to gel blot analysis using an anti-MBP antibody, which revealed bands of about 70 kDa, the expected size of the MBP-SylA fusion protein (data not shown). To test whether the MBP-SylA fusion protein was functional *in vivo,* pMEKmSylA was transformed into the *Pss* B301D-R *ΔsylA* deletion mutant and tested for complementation of the syringolin A-negative phenotype using a rice infiltration assay. We have previously established that infiltration of syringolin A or syringolin A-synthesizing bacteria into rice leaves results in the accumulation of *Pir7b*

transcripts, whereas syringolin A-negative strains or mutants do not induce this gene. (Hassa et al., 2000; Ramel et al., 2009; Wäspi et al., 1999; Wäspi et al., 1998b). Gel blot analysis of RNA from infiltrated rice leaves revealed that transformation of the ΔsylA mutant with pMEKmSylA complemented the mutant and led to strong *Pir7b* transcript accumulation, whereas no *Pir7b* transcripts were detected in leaves infiltrated with the ΔsylA mutant with or without the empty control vector (Figure 5A). This result confirmed that the MBP-SylA fusion protein was functional *in vivo.*

Binding of the MBP-SylA fusion protein to *sylB/sylC* promoter fragments was examined using a magnetic DNA affinity enrichment (MDAE) approach. A 244 bp fragment containing the intergenic region between the *sylB* and *sylC* genes as well as 18 bp and 5 bp of the *sylB* and *sylC* ORF was amplified by PCR using a biotinylated primer. As a negative control, a biotinylated internal 120 bp fragment of the *sylC* coding region was generated. The purified DNA fragments were immobilized on streptavidin-coated magnetic beads, which were then incubated with crude protein extracts from *Pss* B301D-R or *E. coli* expressing the MBP-SylA fusion protein or MBP from the empty vector alone. Bound proteins were eluted and subjected to gel blot analysis using MBP-specific antibodies. A band with the expected size of the fusion protein of about 70 kDA was observed with the *sylB*/*sylC* promoter probe but not with the negative control probe (Figure 5B). Extracts from bacteria carrying the empty vector producing MBP alone (51 kDa) resulted sometimes in a faint background band in the expected size range. Next, a 64 bp long subfragment (underlined in Figure 2) encompassing the sequence upstream of position -66 relative to the *sylB* gene and upstream of – 91 relative to the *sylC* gene, and which contains the putative RhlR-like binding site, was tested in MDAE experiments. As shown in Figure 5B (right panel), MBP-SylA bound to this fragment as well. Identical results were obtained with protein extracts from *Pss* B301D-R and *E. coli* harbouring the respective MBP::SylA

expression plasmids. These results strongly suggest that SylA is directly activating the *sylB* and *sylC* genes.

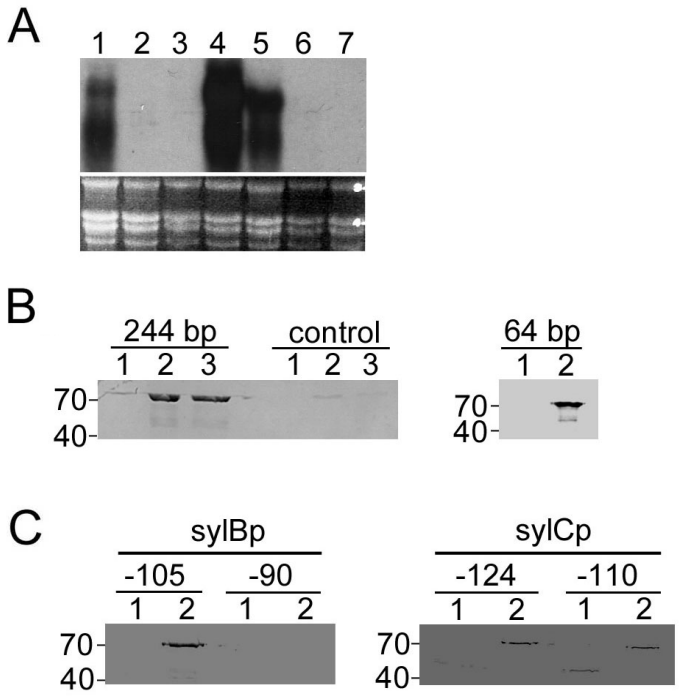


Figure 5. Promoter binding assays with MBP-SylA and MBP-SalA fusion proteins.

A, RNA gel blot analysis of *Pir7b* transcript accumulation in rice leaves 16 h after infiltration of the wild-type *Pss* B301D-R strain (lane 1), the *ΔsylA* mutant (lane 2), *ΔsylA* mutant containing the empty vector pMEKm12 (lane 3), the *ΔsylA* mutant complemented with pMEKmSylA (lane 4), the *salA* insertion mutant DSL7 complemented with pMEKmSalA (lane 5), DSL7 containing the empty vector pMEKm12 (lane 6), and DSL7 (lane 7). Top panel: Autoradiogram; bottom panel: EtBr-stained gel before blotting. The gel blot was probed with a radiolabeled *Pir7b* cDNA probe.

B, Gel blot analysis of protein eluates after MDAE using an MBP-specific antibody for immunodetection. Left panel: A 244 bp *sylB/sylC* promoter fragment or a 120 bp control fragment derived from the *sylC* coding region was bound to magnetic beads and incubated with extracts of *Pss* B301D-R carrying the empty vector (lane 1), MBP-SylA encoding pMEKmSylA (lane 2), and a 1:1 mixture of extracts from bacteria carrying pMEKmSylA or pMEKmSalA, respectively (lane 3). Right panel: MDAE with extracts from *E. coli* carrying the empty vector (lane 1) or pMalSylA (lane 2) using a 64 bp subfragment.

C, MDAE with *E. coli* extracts (lane 1, empty vector; lane 2, pMalSylA) using the indicated *sylB* (left panel) and *sylC* (right panel) promoter deletion fragments. The position and size in kDa of marker proteins is indicated on the left of panels in **B** and **C.**

We also assayed fragments corresponding to the -105, -90, and -75 *sylB* promoter deletions. While the -105 *sylB* promoter fragment bound the MBP::SylA fusion protein, the -90 fragment (Figure 5C, left panel) and the -75 fragment (not shown) did not. This result is in agreement with the results from the promoter activity assays (Figure 3A) and further suggests that the putative RhlR-like binding element, which is present in the -105 deletion, but severely truncated in the -90 deletion (Figure 2), is important for *sylB* activation by the *sylA* gene product. The corresponding results with *sylC* deletion fragments revealed that the -124 deletion fragment only weakly bound the MBP::SylA fusion protein, while the -110 fragment weakly bound the fusion protein equally well as the MBP protein alone, i. e. unspecifically (Figure 5C, right panel). Results with the -95 and -82 fragments were similar to the ones obtained with the -110 deletion.

3.3.5 SalA is an upstream activator of syringolin A biosynthesis

First, we examined the activity of the *sylA* promoter by constructing a *sylA*::*lacZ* translational fusion gene (psylAp-lacZ) that encompassed the 445 bp between the *sylA* translation initiation codon and the next upstream gene (which is highly conserved in *P. syringae* strains that lack a syringolin synthetase gene cluster, like e.g. *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola*) and the first 86 bp of the *sylA* ORF. β-gal assays of wild-type transformed with psylAp-lacZ and grown under still culture conditions showed an activity of around 300 Miller units (MU), while the same construct in the ΔsylA background led to an about 50% higher activity (Table 2). Therefore, we checked whether this might indicate negative feed-back control of SylA on its own gene promoter by placing the *sylA* gene under the control of the *lacZ* promoter onto psylAp-lacZ to yield pOEAA. However, β-gal assays of the B301D-R wild-type strain carrying pOEAA exhibited similar activities as the one carrying psylAp-lacZ (data not shown), thus not confirming the existence of a negative feed-back loop. Interestingly, *sylA::lacZ*

fusion genes containing only 11 bp of the *sylA* ORF did not result in detectable β-gal activity.

Evidence from a microarray hybridization experiment reported in the literature (Lu et al., 2005) suggested that the *sylD* gene belonged to the regulon controlled by the HTH LuxR-type transcription factor SalA, which was reported to positively regulate syringomycin biosynthesis (Lu et al., 2002a; Wang et al., 2006). Therefore, we tested whether SalA was also involved in syringolin A biosynthesis. Thus, psylAp-lacZ, psylBp-lacZ and psylCp-lacZ reporter plasmids were transformed into the *salA* transposon insertion mutant DSL7 (Lu et al., 2002a) and β-gal activities were determined. As shown in Table 2, the activities of the *sylB* and *sylC* promoters were strongly decreased in the *salA* mutant as compared to the wild type, indicating that the *salA* gene was necessary for expression of *sylB* and *sylC*. Overexpression of *sylA* (using plasmid pOEAB and pOEAC) in the *salA* mutant background led to a dramatic increase of *sylC* and *sylB* reporter, indicating that *salA* acts upstream of *sylA*. Consistent with this, syringolin A accumulation in conditioned media of the *salA* insertion mutant DSL7 was abolished, but was restored by overexpression of *sylA* in this mutant (data not shown).

The *salA* gene under the control of the *lacZ* promoter was inserted into the psylAp-lacZ, psylBp-lacZ and psylCp-lacZ reporter plasmids to yield pOESalAA, pOESalAB, and pOESalAC, respectively. When transformed into the *ΔsylA* mutant, pOESalAB and pOESalAC led to similarly low β-gal activities as psylBp-lacZ and psylCp-lacZ in the same genetic background, showing that overexpression of *salA* had no effect on the *sylB* and *sylC* promoters in the *ΔsylA* background (data not shown). In contrast, overexpression of *salA* in wild-type bacteria transformed with pOESalAA consistently increased the activity of the *sylA* promoter about two- to three-fold above the wild-type levels of ca. 300 MU, while the *sylA* reporter construct psylAp-lacZ led to β-gal activities of 172 MU in the *salA* mutant background (Table 2). The *sylA* reporter

plasmid psylAp-lacZ in wild-type bacteria led to blue staining of colonies in *N. benthamiana*, whereas no staining was detected in plants infiltrated with the *salA* mutant DSL7 carrying psylAp-lacZ (Figure 4B).

Table 2. Effect of *salA* on *sylA, sylB, and sylC* reporter gene activity

Strain [a]	**Reporter**	**β-gal activity** [b]	**Comment**
WT (psylAp-lacZ)	sylA	299 ± 28	
ΔsylA (psylAp-lacZ)	sylA	445 ± 25	
WT (psylBp-lacZ)	sylB	763 ± 59	n=9
WT (psylCp-lacZ)	sylC	1237 ± 33	n=9
DSL7 (psylBp-lacZ)	sylB	76 ± 9	*salA* mutant, n=9
DSL7 (psylCp-lacZ)	sylC	209 ± 14	*salA* mutant, n=9
DSL7 (pOEAB)	sylB	7778 ± 218	*salA* mutant, sylA overexpr.
DSL7 (pOEAC)	sylC	6717 ± 340	*salA* mutant, sylA overexpr.
WT (pOESalAA)	sylA	793 ± 75	*salA* overexpression
DSL7 (psylAp-lacZ)	sylA	172 ± 16	*salA* mutant

[a] WT = wild type
[b] β-Galactosidase (β-Gal) activity. ± standard error (n > 15) in Miller units (MU).

These findings suggest that SalA activates directly or indirectly the *sylA* gene, whose product then activates the *sylB* and *sylC* genes. Next, we tested whether SalA directly binds to the *sylA* promoter region using MDAE. The *salA* gene was cloned into pMEKm12 as a translational fusion to MBP, resulting in pMEKmSalA. This plasmid complemented the *salA* mutant DSL7 with regard to syringolin A production as monitored by *Pir7b* transcript accumulation upon infiltration of transformed bacteria into rice leaves, thus confirming that the MBP-SalA fusion protein was functional *in vivo* (Figure 5A). However, no binding of MBP-SalA to the putative *sylA* promoter fragment encompassing 445 bp of sequence upstream of the *sylA* translation initiation codon

and the first 86 bp of the coding region was observed (data not shown). Binding to this fragment was also not observed after incubation with a 1:1 mixture of extracts containing MBP-SalA and MBP-SylA, respectively.

Because these results may indicate that the action of SalA on the *sylA* gene is indirect, we considered two other genes encoding HTH LuxR-type regulatory proteins, *syrF* and *syrG*, as candidates possibly involved in *syl* gene regulation. *SyrF* was reported to be directly activated by the SalA regulator and to activate in turn genes controlling syringomycin biosynthesis (Wang et al., 2006). *SyrG* is physically located adjacent to the *salA* gene and was reported to exhibit a twofold reduced expression level in a *salA* mutant. In addition, a *syrG*-negative mutant exhibited reduced virulence on cherry fruits (Lu et al., 2002a).

We thus generated *syrF* and *syrG* insertion mutants (SyrF_KO and SyrG_KO) in which the HTH LuxR-type DNA-binding domain of the putative gene products were interrupted. Upon transformation of the *sylA* and *sylC* reporter plasmids psylAp-lacZ and psylCp-lacZ into these mutants, similar β-gal activities were observed as in the wild type carrying the same plasmids. Furthermore, rice infiltration assays with the *syrF* and *syrG* mutants exhibited wild-type *Pir7b* transcript levels as determined by RNA gel blot analysis (data not shown). Thus, there is no evidence that *syrF* and *syrG* play a role in the regulation of syringolin A biosynthesis.

3.3.6 Why does syringolin A accumulate in still but not in shaken cultures?

Syringolin A can be isolated from conditioned media of *Pss* B301D-R grown under still culture conditions in SRM_{AF} minimal medium (Wäspi et al., 1998a). Typically, 20-40 mg/l can be obtained after 7 to 10 days at 28° C. In conditioned rich media (shaken and unshaken) or in shaken SRM_{AF} cultures, syringolin A accumulates only to concentrations barely detectable by direct HPLC analysis (< 2 mg/l). Overexpression of the plasmid-borne *sylA* gene under the control of the *lacZ* promoter results in greatly

increased syringolin A production both in still and shaken SRM_{AF} cultures (typically 200-400 mg/l). Correspondingly, *sylA*-overexpressing *sylB::lacZ* and *sylC::lacZ* reporter plasmids (pOEAB and pOEAC) resulted in high β-gal activities in shaken cultures, in contrast to psylBp-lacZ and psylCp-lacZ not overexpressing *sylA* (data not shown). One obvious difference between still and shaking culture conditions is the cell density reached. Typically, in still cultures a plateau at an OD_{600} of 0.4-0.5 is reached after 3-4 days of growth at 28°C which is maintained until day 6 or 7 but declines thereafter. Because shaken cultures in SRM_{AF} reach much higher cell densities (up to an OD_{600} of 4 after 4 days, and declining thereafter) and are much better aerated, we considered the possibility that syringolin A production might either be under negative quorum sensing (QS) control, or be inhibited by high oxygen concentration.

To test the first possibility, a markerless quorum sensing-negative mutant was constructed in *Pss* B301D-R, in which the orthologs of the adjacent *ahlI* autoinducer and *ahlR* regulator genes of the closely related *Pss* B728a strain (Quinones et al., 2004) were deleted. Transformation of the *sylB* and *sylC* reporter plasmids psylBp-lacZ and psylCp-lacZ into this mutant revealed reporter gene activities not significantly different from those observed in the wild-type background under both still and shaking SRM_{AF} cultures (data not shown). In addition, HPLC analysis of conditioned media from mutant and wild-type cultures contained similar syringolin A concentrations under all conditions (not shown). We thus conclude that syringolin A biosynthesis is not dependent on the *ahlI/ahlR* quorum sensing system.

To examine the dependency of syringolin A production *in vitro* on oxygen concentration, *sylC* reporter activities (psylCp-lacZ in the *Pss* B301D-R wild type) were compared in shaken SRM_{AF} cultures under normal and low oxygen conditions. Cultures were grown for 64 h under normal shaken conditions, i.e. culture flasks were capped with a lid allowing gas exchange. Thereafter, half of the culture flasks were flushed with N_2 and sealed with parafilm to reduce gas exchange. All cultures were then shaken for

another 46 h. As shown in Figure 6, *sylC::lacZ* reporter activity increased significantly upon oxygen limitation, indicating that oxygen concentration does play a role in the regulation of syringolin A biosynthesis.

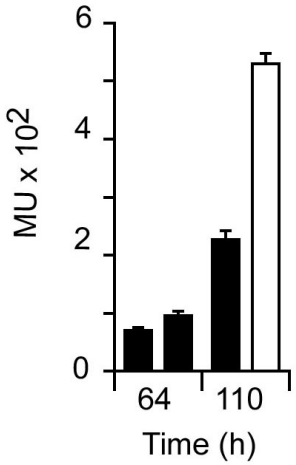


Figure 6. Effect of oxygen concentration on *sylC::lacZ* reporter activity.
Promotor activities were measured in *Pss* B301D-R bacteria transformed with the psylCp-lacZ reporter construct. Cultures were first shaken for 64 h under aerated conditions. Then, oxygen was excluded from half of the cultures for another 46 h of shaking incubation (open bar), while the other half was shaken under aerated conditions (black bar). Average β-gal activities in Miller units (MU) are given (n=5). Error bars indicate the standard error of the mean.

A well-known regulator of responses to limiting oxygen concentrations is the *E. coli* Fnr protein and its *P. aeruginosa* homologue Anr (Blumer and Haas, 2000; Green et al., 2009; Sawers, 1999). Under anaerobic conditions, Fnr forms a dimer that binds to DNA and can either activate or repress genes (Bauer et al., 1999; Melville and Gunsalus, 1996). The only close homologue of Anr in *Pss* B728a is YP_236495 (86% identity/94% similarity) encoded by Psyr_3425 (GeneID 3368952). In order to examine the role of the Psyr_3425 orthologue in the regulation of syringolin A biosynthesis, a marker-less deletion mutant was generated in the *Pss* B301D-R background, which then was transformed with the psylCp-lacZ and psylAp-lacZ reporter genes. Reporter

activities of the respective plasmids in the mutant background were not significantly different from the ones measured in the wild-type background under both still and shaken culture conditions (data not shown). Furthermore, infiltration of wild-type and mutant strains into *N. benthamiana* leaves led to indistinguishable β-gal activity staining (Figure 4C). In addition, both the wild-type and the Psyr_3425-negative strains led to essentially the same *Pir7b* transcript levels upon infiltration into rice leaves. In summary, these results indicate that the Anr orthologue of *Pss* B301D-R does not play a role in the regulation of syringolin A biosynthesis.

3.4 Discussion

Syringolin A biosynthesis can be conferred to the saprophytic *P. putida* by transformation with a cosmid harbouring the *sylA-E* gene cluster, indicating that no other specific genes are necessary (Ramel et al., 2009). Here we presented evidence that *sylC, sylD,* and *sylE* form an operon. The activity of the *sylCDE* operon and the *sylB* gene on the opposite strand depends on the *sylA* gene and is greatly increased upon overexpression of the latter.

Overlapping deletions of the *sylB/sylC* intergenic region allowed to delimit minimal promoter fragments necessary to drive *sylB::lacZ* and *sylC::lacZ* reporter genes. The 226 bp full length *sylB* promoter (comprising 221 bp intergenic DNA and 5 bp *sylC* coding region) translationally fused to the *lacZ* gene resulted in an activity of about 700 MU. Essentially the same activities were also observed with the shorter fragments of 153, 117, and 105 bp. Shorter fragments of 90, 75, 67, and 57 bp resulted in the same basal activity of about 200-300 MU, suggesting that the sequence between 105 and 90 bp contains elements important for promoter activity. These results were corroborated *in planta* upon infiltration of bacteria harboring these reporter constructs.

Blue colonies inside the leaf appeared only with promoter deletions shown to be active in *in vitro* cultures, while no blue staining was detected with the reporter genes resulting in basal activities *in vitro.* This 15 bp region overlaps with a putative binding site for *P. aeruginosa* RhlR, the HTH-LuxR-type response regulator of a quorum sensing system in *P. aeruginosa*, from position -100 to -85 relative to the *sylB* translational start site (5'-CTGTAAAAAATAGCAG on the *sylB* strand). As SylA also exhibits a putative HTH LuxR-type DNA binding domain, it seems likely that this site is important for *sylB* gene activation. Indeed, this element (5'-CTGCTATTTTTTACAG on the *sylC* coding strand) appears also to be essential for *sylC* promoter activity. Although the shortest active *sylC* promoter deletion (124 bp of upstream sequence) contained only a severely truncated version, a new high-scoring putative binding site was fortuitously created together with flanking vector sequence, which likely explains the activity of this fragment *in vitro* and *in planta*. These data are essentially in agreement with the MDAE experiments using promoter fragments and protein extracts from *E. coli* and *Pss* B301D-R which produce an MBP::SylA fusion protein. It is worthwhile to mention that all our efforts to perform MDAE experiments using affinity-purified MBP::SylA proteins, or to perform electrophoretic mobility shift assays with them, remained unsuccessful, which may indicate that the MBP::SylA fusion protein either is instable, or that binding only occurs in the presence of one or more additional factors which, however, would have to be present in extracts of both *E. coli* and *Pss* B301D-R.

Whereas the results of the deletion analysis suggest that, apart from the small region containing the RhlR-like binding motif, no other upstream sequence element is important for *sylB* transcription, the consistently higher activity of the 239 bp (full length) *sylC* promoter as compared to the -175 and -124 deletions (Figure 2) suggests that other sequence elements enhancing *sylC* transcriptional activity reside between positions -239 and -124. Virtual Footprint analysis revealed, as the only notable feature we observed in this region, putative *P. aeruginosa* integration host factor (IHF) binding

sites (5'-CAATGCGT between -195 and -188 on the *sylC* coding strand; 5'-CAACGCAT between -186 and -193 on the bottom strand relative to the *sylC* translation initiation codon). IHF can induce DNA bends (U-turns) at its binding site and is known to influence transcription (Dillon and Dorman, 2010; Huo et al., 2009; Macchi et al., 2003). It remains presently unknown if these sites play a role in *sylC* transcriptional regulation.

We have previously established that syringolin A biosynthesis is controlled by the GacS/GacA two-component system and is abolished in a *gacS* (formerly designated *lemA*) mutant (Reimmann et al., 1995; Wäspi et al., 1998a). The GacS/GacA global regulatory system has been found in numerous bacteria, including plant pathogens, where it was shown to control a plethora of phenotypes, among them pathogenicity, virulence, toxin production, and ecological fitness (Chatterjee et al., 2003; Heeb and Haas, 2001; Hirano et al., 1997; Hirano and Upper, 2000; Hrabak and Willis, 1992; Hrabak and Willis, 1993; Willis et al., 1990). In the bean pathogen *Pss* B728a, some of the phenotypes observed in a *gacS* mutant, including loss of syringomycin production and lesion formation, could be restored by overexpressing the *salA* gene, whose expression was shown to depend on the GacS/GacA system (Kitten et al., 1998).

Here we showed that syringolin A biosynthesis also is mediated by the *salA* gene product. Inactivation of *salA* resulted in negligible syringolin A production in *in vitro* cultures and to only basal activity of the *sylB::lacZ a*nd *sylC::lacZ* reporter genes. *SalA* acts upstream of *sylA* in the regulatory cascade because overexpression of *sylA* in the *salA* mutant resulted in very high activities of the *sylB::lacZ a*nd *sylC::lacZ* reporter genes. The moderate *sylA::lacZ* reporter activity observed in the wild type was reduced to about 50% in *in vitro* cultures of the *salA* mutant, whereas *in planta*, no blue staining was observed. In contrast, overexpression of *salA* led to a 2-3 fold increase of the *sylA::lacZ* reporter activity above wild-type levels, indicating that SalA directly or indirectly activates the *sylA* gene. We were not able to demonstrate direct binding of a

MBP-SalA fusion protein, which was functional *in vivo*, to the *sylA* promoter. This may be due to experimental circumstances, or, alternatively, it may indicate that the activation is indirect via the activation of one or more additional transcription factor(s). The most likely candidates we considered were the *syrF* and *syrG* genes. Both encode HTH LuxR-type regulators, reside at the border of the large syringomycin/syringopeptin gene cluster, and were shown by microarray analysis to belong to the SalA regulon (Lu et al., 2005). In addition, SyrF was shown to activate *syr-syp* genes and bind to a *syrB1* promoter fragment, while SalA was necessary for *syrF* activity and bound to its promoter region (Wang et al., 2006). However, both the *syrF* and *syrG* mutants we generated showed wild-type behavior with regard to syringolin A production *in planta* and *sylA* and *sylC* reporter gene activity *in vitro*, and therefore were ruled out as regulators of syringolin A biosynthesis. As the microarray used by Wang et al. (Wang et al., 2006) only contained 95 gene probes, the SalA regulon may well comprise additional unknown genes.

Because in wild-type bacteria, syringolin A accumulated to considerable amounts in conditioned SRM_{AF} media only under still culture conditions, where lower cell densities are reached and oxygen availability is limited, we considered quorum sensing and oxygen concentration as possible negative regulators of syringolin A biosynthesis. However, an *ahlI/ahlR* deletion mutant exhibited a wild-type phenotype in both still and shaken cultures, indicating these genes are not involved in syringolin A biosynthesis. Similarly, quorum sensing was reported not to be involved in the regulation of syringomycin biosynthesis (Dumenyo et al., 1998; Kinscherf and Willis, 1999).

In contrast to the quorum sensing system, oxygen concentration seems to play a role in syringolin A biosynthesis as evidenced by the fact that the *sylC::lacZ* reporter gene resulted in a 2-3 fold higher β-gal activity in shaken cultures under oxygen-limiting conditions as compared to well aerated cultures. Because in *P. aeruginosa*, the Fnr-type oxygen sensor Anr is known to mediate the regulation of many pathways activated

or repressed by low oxygen tension (Green et al., 2009; Trunk et al., 2010), we deleted the *anr* orthologue in *Pss* B301D-R. However, no significant effect of this deletion was detected, neither on *sylA::lacZ* and *sylC::lacZ* reporter gene readouts under all culture conditions and upon infiltration into *N. benthamiana*, nor on *Pir7b* transcript levels upon infiltration into rice leaves.

The potential of oxygen limitation as an inducer for the production of secondary metabolites by microbes in culture has long been recognized (Clark et al., 1995; Rollins et al., 1988). In the case of *P. syringae* pv. *syringae*, which is a leaf pathogen, the biological significance of the apparent oxygen sensitivity of syringolin A biosynthesis is not obvious. During the day, when stomata are open and photosynthesis takes place, oxygen is hardly limiting, even though the bacteria seem to form dense localized colonies (see Figure 4), which may develop biofilm-like properties. During the night, when stomata are closed and no photosynthesis occurs, this may be different. However, it is also possible that oxygen limitation *in vitro* activates a pathway leading to syringolin A biosynthesis which *in planta* is activated by other stimuli. Clearly, the picture obtained of the regulation of syringolin A biosynthesis remains incomplete. In order to identify additional genes involved, a more complete exploration of the *salA* regulon using *Pss* B728a whole genome microarrays and the development of an efficient screening method for the identification of syringolin A-negative mutants would be helpful.

3.5 Material and methods

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. Unless otherwise stated, bacteria were grown in LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) containing the appropriate antibiotics (100 µg/mg ampicillin, 50 µg/ml gentamicin, 50 µg/ml kanamycin, or 10 µg/ml tetracyclin) on a shaker at 28°C (*Pss*) or 37°C (*E. coli*).

Syringolin A-inducing culture conditions

Five-ml-cultures of *Pss* were grown o/n in SRM_{AF} medium (1% D-glucose, 0.1% D-fructose, 100 µM arbutin, 0.4% L-histidine, 0.8 mM $MgSO_4$, 10 µM $FeCl_3$, 0.8 mM potassium phosphate, pH 7 (Gross and DeVay, 1977; Mo and Gross, 1991b)) and 10 µg/ml tetracyclin, if appropriate, at 28°C on a shaker (220 rpm/min) o/n. The density was adjusted to 0.2 OD_{600} and 1 ml aliquots were used to inoculate 100 ml SRM_{AF} cultures in 250 ml Erlenmeyer flasks, which were incubated at 28°C for 5-7 days without shaking.

For low oxygen condition experiments, 100-ml-cultures were grown in 250 ml Erlenmeyer flasks on a shaker (220 rpm) for 64 h. Then, culture flasks were flushed with N_2, the cap was removed and the flask was sealed with parafilm to impede gas exchange. Cultures were then shaken for another 46 h.

Detection of syringolin A production

Syringolin A in conditioned media was detected by HPLC analysis as described (Ramel et al., 2009). Syringolin A production by bacteria *in planta* was detected by infiltrating bacteria into leaves of 2-3-weak-old rice (*Oryza sativa* cv. Loto) plants and analyzing accumulation of *Pir7b* transcripts by RNA gel blots 16 h after infiltration as described

(Ramel et al., 2009). *Pir7b* encodes an esterase (Wäspi et al., 1998b) whose corresponding transcript accumulates only upon infiltration of *Pss* producing syringolin A. Syringolin A was originally isolated based on *Pir7b* induction (Wäspi et al., 1998a).

Extraction and analysis of RNA from bacteria

Pss B301D-R transformed with pOEAC was grown on a shaker in 50 ml SRM_{AF} medium o/n at 28°C. Bacteria were pelleted and resuspended in 1.6 ml of cold trizol solution (38% phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, 5% glycerol (v/v)). After centrifugation at 20'000 x g for 10 min at 4°C, the supernatant was recovered and vigorously shaken with 300 µl of chloroform for 15 min at room temperature. After centrifugation for 8 min at 20'000 x g at 4°C, 800µl of the supernatant was mixed with 400 µl each of isopropanol and 0.8 M sodium citrate/1.2 M NaCl solution. The mixture was incubated at -20°C o/n and RNA was pelleted by centrifugation for 8 min at 12'000 x g, washed with 75 % ethanol, air dried, and dissolved in 60 µl diethylpyrocarbonate (DEPC)-treated water. RNA gel blot analysis, ^{32}P-labeling of probes, and hybridization were performed according to standard procedures (Ausubel et al., 1987). The probe fragments were amplified from *sylC* and *sylE* genes with primers sylC_N_f, sylC_N_r and sylE_N_f, sylE_N_r, respectively (Suppl. Table 2).

RT-PCR reactions with 1.5-2 µg of total RNA were performed using the OneStep RT-PCR Kit (QIAGEN, AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol using primers RT_sylCD_f, RT_sylCD_r (for the *sylC/sylD* intergenic region), RT_sylDE_f, and RT_sylDE_r (for the *sylD/sylE* intergenic region).

Construction of mutants

Gene fragments of *syrF* (accession no. AF372703; ORF position 59-889; fragment 127-802) and *syrG* (accession no. AF372703; ORF position 6136-6924; fragment 6249-6827) were amplified with primer pairs syrF_KO_f/r and syrG_KO_f/r (Suppl. Table 2) from genomic DNA of *Pss* B301D-R and cloned into the suicide vector pJQ200KS using the restriction sites *BamH*I and *Xho*I. Recombinant plasmids were transformed into *E.coli* S17-1 and mobilized into *Pss* B301D-R. Colonies carrying chromosomally integrated plasmids were selected for gentamicin resistance and plasmid insertions at the correct site were verified by PCR as described (Amrein et al., 2004).

The construction of the marker-less ΔsylA mutant has been described (Ramel et al., 2009). Marker-less ΔahlI/ahlR and Δanr mutants in the *Pss* B301D-R background were constructed in an analogous way, whereby primers (Suppl. Table 2) were designed according to the genome sequence of *Pss* B728a (accession no. NC_007005.1, *ahlI* geneID 3367123, *ahlR* geneID 3367124, *anr* geneID 3368952 (Psyr_3425 orthologue). In each case, flanking fragments were amplified using primer pairs P1/P2 and P3/P4 (Suppl. Table 2), which were joined by overlap extension PCR using primer pair P1/P4. Mutants were verified by PCR using primers flanking the deleted sequences.

Construction of promoter-reporter fusion genes

Syl promoter fragments were amplified by PCR from B301D-R genomic DNA. Primer sequences (Suppl. Table 2) were designed according to the sequence of the *syl* gene cluster (accession no. AJ548826). All promoter fragments were flanked by *EcoR*I and *Pst*I sites and cloned into pME6014, resulting in translational fusions to the *lacZ* gene named psylAp-lacZ, psylBp-lacZ, and psylCp-lacZ. The *sylA*-overexpressing *sylA* and *sylB* reporter plasmids pOEAA and pOEAB were constructed analogously to pOEAC,

as described (Ramel et al., 2009). In short, the *sylA* ORF was put under the control of the *lacZ* promoter and placed onto the reporter plasmids psylAp-lacZ, psylBp-lacZ, and psylCp-lacZ, respectively, in opposite orientation. Similarly, the *salA*-overexpressing *sylA* (pOEsalAA)*, sylB* (pOEsalAB)*,* and *sylC* (pOEsalAC) reporters were constructed by amplifying the *salA* ORF (accession no. AF372703, position 7960-8829) from B301D-R genomic DNA using primers salA_OE_f and salA_OE_r (Suppl. Table 2). The PCR product was cloned into pME6001^{k} behind the *lacZ* promoter to yield pOESalA^{k}, from which the *lacZp-salA* fusion gene was amplified using primers SalA_OE_r and lacZp_OL_sylAp (or lacZp_OL_sylBp or lacZp_OL_sylCp, respectively). Then, the *sylA, sylB,* and *sylC* promoter fragments were amplified from psylAp_lacZ, psylBp_lacZ, and psylCp_lacZ, respectively, with the respective primer pairs syl[A/B/C]p_OL_lacZp / syl[A/B/C]p_r. These fragments were joined to the corresponding *lacZp-salA* fusion fragments by overlap extension PCR with primer pairs salA_OE_r / syl[A/B/C]p_r (Suppl. Table 2), digested with *Eco*RI and *Pst*I, and cloned into the polylinker of pME6014. All constructs were verified by sequencing.

Quantitative β-galactosidase assays

Quantitative β-gal assays were performed as described (Miller, 1972) with some modifications. Samples bacterial cultures were harvested from 72 to 168 h post inoculation (hpi) in intervals of about 24 h. 1 ml Z-buffer (60 mM Na_2HPO_4 x $2H_2O$, 40 mM NaH_2PO_4 x H_2O, 10 mM KCl, 1 mM $MgSO_4$ x 7 H_2O, 50 mM β-mercaptoethanol), 20 µl 0,1% SDS, 40 µl chloroform and 100-300 µl bacterial culture were mixed by inversion. The suspension was centrifuged at 1000 x g for 5 min. To 1 ml supernatant, 60 µl of reagent solution (60 mM Na_2HPO_4 x $2H_2O$, 40 mM NaH_2PO_4 x H_2O, 1.4% (w/v) ortho-nitrophenyl-β-D-galactoside) were added. The reaction was stopped by adding 150 µl 1M Na_2CO_3. Absorption was measured at OD_{420} and Miller units (MU) were calculated according to the formula MU = 1000 x OD_{420} x 1.21 / t x V x OD_{600} x 1.7 (t =

reaction time in min; V= volume of bacterial suspension added; formula adapted for the reagent volumes used here). Experiments were repeated independently two or three times, each with three to five replicate cultures. Data are usually shown as the average from the measurements at 96 hpi, 120 hpi, and 144 hpi, when values normally have reached a plateau.

β-galactosidase assay *in planta*

Three-weak-old *Nicotiana benthamiana* plants grown at 25°C in a greenhouse under a 16 h light/8 h dark regime were infiltrated with a suspension of bacteria transformed with reporter constructs at an OD_{600} of 0.0002 (ca. 10^5 bacteria/ml) using a syringe without a needle. Leaves were harvested after 72 to 96 hpi. Leaf discs (Ø 1cm) were punched out from infiltrated areas and vacuum infiltrated with 1% glutaraldehyde in potassium phosphate buffer (0.2 M $NaPO_4$, pH 7) to inactivate the endogenous β-galactosidase of the plants (Teeri et al., 1989). Leaf discs were washed three times in potassium phosphate buffer and incubated overnight with 1 mM 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal). Stained discs were washed once in potassium phosphate buffer and incubated in acetone: methanol (1:3) for 3 h at 37°C to remove chlorophyll. After a final wash in 100% ethanol, leaf discs were arranged on glass slides in 50% glycerol for observation under the light microscope.

MBP-SylA and MBP-SalA fusion proteins

SylA and *salA* genes were amplified by PCR using primer pairs sylAFP_f/r and salAFP_f/r (Suppl. Table 2), respectively, and cloned into the vectors pMal-c2X and pMEKm12 to yield pMalSylA and pMEKmSylA for expression in *E. coli* and *Pss* B301D-R, respectively. After verification by sequencing they were transformed into *E.coli* DH10B and *Pss* B301D-R. Bacterial cultures (3 x 200 ml) in LB with the appropriate antibiotic were grown to an OD_{600} of 0.5-1 before expression of the fusion gene was

induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.3 mM for *E. coli* and 5 mM for *Pss*. Cultures were further incubated for 5 (*E. coli*) and 7 (*Pss*.) h before bacteria were pelleted by centrifugation (4000 x g, 20 min, 4°C) and resuspended in a total of 22.5 ml binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.01% Triton X-100). The suspension was supplemented with protease inhibitors (1 tablette per 10 ml of Complete Mini Protease Inhibitor Cocktail, Roche Applied Science, Rotkreuz, Switzerland). Cells were broken with a French press and cell debris was removed by centrifugation (10000 x g, 30 min, 4°C). The protein concentration in the supernatant was estimated by measuring the absorption at 280 nm. On average, extracts contained ca. 35 mg/ml (*E.coli*) and 25 mg/ml (*Pss*) protein. To verify the correct size of fusion proteins (MBP-SylA, 69 kDa; MBP-SalA, 74 kDa; MBP, 51 kDa), protein extracts were analysed on protein gel blots using an anti-MBP antibody (New England Biolabs, Ipswich, USA) according to standard procedures (Ausubel et al., 1987).

Magnetic DNA affinity enrichment

Magnetic DNA affinity enrichment (MDAE) was carried out with minor modifications as described (Koebnik et al., 2006). To amplify biotinylated promoter probes, the biotinylated primers listed in Suppl. Table 2 were used. Ten pmol of purified biotinylated DNA in 200 µl H_2O was added to an equal volume of magnetic beads (Dynabeads; Invitrogen, USA) suspension prepared as described (Koebnik et al., 2006). The suspension was incubated with slight agitation for 2 h at room temperature, washed three times for 5 min with wash buffer (10 mM Tris-HCl, pH 7.5, 2 M NaCl), and resuspended in 1,1 ml of binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.01% Triton X-100) containing 18 mM maltose and a 10-fold (w/w) excess of salmon sperm DNA (Sigma-Aldrich, Buchs, Switzerland) over fragment DNA. Finally, the suspension was mixed with 900 µl of

whole protein extract from *Pss* B301D-R or *E. coli* overexpressing MBP, MBP-SylA or MBP-SalA protein. The mixture was gently agitated for 45 min at room temperature. Beads were then washed with binding buffer (3 times 5 min; in the second wash step competitor DNA was added) and proteins were eluted twice with 100 µl elution buffer (20 mM Tris-HCl, pH 7.5, 2 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.01% Triton X-100). The two eluates were combined and concentrated to about 40 µl using Vivaspin 500 (MWOC 10'000, Sartorius AG, Göttingen, Germany). Proteins were subjected to gel blot analysis using anti-MBP antibody according to standard procedures (Ausubel et al., 1987).

Author contributions

CR planned and performed most of the experiments. NB, MM, MH, and DS were involved in the construction of some mutants and some β-gal assays. CR and RD designed research and wrote the paper.

Acknowledgements

We would like to thank C. Reimmann, D. Haas, and D. C. Gross for strains and vectors, and Zsuzsa Hasenkamp for technical assistance. Financial support from the Swiss National Science Foundation (grants 3100A0-115970 and 31003A-134936) and the University of Zurich is gratefully acknowledged.

Supplementary tables

Supplementary Table 1. Bacterial strains and plasmids

Strains	Genotype / Relevant characteristics	Reference
E. coli **:**		
DH10B	F$^-$ *endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZ*ΔM15 *araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC)* λ$^-$.	(Grant et al., 1990)
XL1-Blue	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44* F'*[::*Tn*10 proAB*$^+$ *lacI^q Δ(lacZ)M15] hsdR17(r$_K^-$ m$_K^+$)*	(Bullock et al., 1987)
S17-1	(*thi pro hsdR recA;* chromosomal RP4 [Tra+ Tc^S Km^S Ap^S]). Used as donor strain in biparental matings.	(Simon et al., 1983)
P. syringae **pv.** ***syringae*** **:**		
B301D-R	Wild type, isolated from pear, Rif^R	(Xu and Gross, 1988)
DSL7	*salA::nptII* insertion mutant in B301D-R, Kan^R	(Lu et al., 2002a)
ΔsylA	*sylA* deletion mutant in B301D-R	(Ramel et al., 2009)
SyrF_KO	*syrF* plasmid insertion mutant in B301D-R, Tet^R	this study
SyrG_KO	*syrG* plasmid insertion mutant in B301D-R, Gm^R	this study
ΔahlI/ahlR	ahlI/ahlR (quorum sensing system) double deletion mutant in B301D-R	this study
Δanr	*Anr* homologue deletion mutant in B301D-R (orthologue of Psyr_3425 of *Pss* B728a).	this study
Plasmids:		
pMal-c2X	Comercial *E.coli* overexpression vector, designed to produce maltose-binding protein fusions. Amp^R	New England Biolabs
pMEKm12	*Pss* overexpression vector, derivative of pMal-c2x. Amp^R, Kan^R	(Lu et al., 2002b)

pME3087	Suicide vector, ColE1 replicon, Tet^R	(Voisard et al., 1994)
pJQ200KS	Suicide vector, p15A ori, Gm^R	(Quandt and Hynes, 1993)
pME6014	*LacZ* reporter plasmid, used for quantitative promoter activity measurements, Tet^R	(Schnider-Keel et al., 2000)
psylAp_lacZ	*sylA* promoter translationally fused to the *lacZ* gene of pME6014, Tet^R	this study
psylBp_lacZ	*sylB* promoter translationally fused to the *lacZ* gene of pME6014, Tet^R	this study
psylCp_lacZ	*sylC* promoter translationally fused to the *lacZ* gene of pME6014, Tet^R	this study
pME6001	*Pss* overexpression vector, Gm^R	(Blumer et al., 1999)
$pME6001^k$	pME6001 derivative, Gm^R, Kan^R	(Ramel et al., 2009)
$pOEA^K$	pME6001 derivative, overexpressing *sylA*, Gm^R, Kan^R	(Ramel et al., 2009)
$pOEsalA^K$	pME6001 derivative, overexpressing *salA*, Gm^R, Kan^R	this study
pOEAC	pME6014 derivative containing a *lacZp::sylA* gene and the *sylCp::lacZ* reporter gene. Tet^R	(Ramel et al., 2009)
pOEAA	pME6014 derivative containing a *lacZp::sylA* gene and the *sylAp::lacZ* reporter gene. Tet^R	this study
pOEAB	pME6014 derivative containing a *lacZp::sylA* gene and the *sylBp::lacZ* reporter gene. Tet^R	this study
pOEsalAAp	pME6014 derivative containing a *lacZp::salA* gene and the *sylAp::lacZ* reporter gene. Tet^R	this study
pOEsalABp	pME6014 derivative containing a *lacZp::salA* gene and the *sylBp::lacZ* reporter gene. Tet^R	this study
pOEsalACp	pME6014 derivative containing a *lacZp::salA* gene and the *sylCp::lacZ* reporter gene. Tet^R	this study

Supplementary Table 2. Primer sequences

Primer name [a]	**Sequence** [b]	**Position** [c] **/Acc. # / Restriction site / Note**
Probes for Northern blot		Acc. # AJ548826
sylC_N_f	GGTATTCTGCGACGGTGTTT	7565
sylC_N_r	TAAAGCCGGAGCACTCTCAT	8170
sylE_N_f	CCACTGCGATGGCCTATATT	22731
sylE_N_r	GTAACGATGATCCGCTGGTT	23238
RT-PCR		Acc. # AJ548826
RT_sylCD_f	TATGCGGATCATGTCGAG	9485
RT_sylCD_r	ATCTGGTGCATGAACCAG	9852
RT_sylDE_f	TCAACCTGCCCGAGATC	22125
RT_sylDE_r	TAGACGATGGCCAGATAG	22502
Constructs for insertion mutants		Acc. # AF372703
syrF_KO_f	cgggatccGTCTAGCGTCGGAGATG	127 / *BamH*I
syrF_KO_r	ccgctcgaGGAACTGCTCTTGACGCA	802 / *Xho*I
syrG_KO_f	cgggatccGCATTTCCTTGACCTGAGC	6249 / *BamH*I
syrG_KO_r	ccgctcgaGAGCTGTTCTTCTTGACGTGC	6827 / *Xho*I
Constructs for deletion mutants		Acc. # NC_007005
P1_ahlI/ahlR	gctctagACACCTCGGTCGATGAC	1822055 / *Xba*I
P2_ahlI/ahlR	**acaatagacaaATAGGCAAATCT**ACTGTAC	1822684
P3_ahlI/ahlR	**agattgcctatTTGTCTATTGT**TTGTTTGCG	1824165
P4_ahlI/ahlR	ttctgcagAGGCCATGTAATAGAAGC	1824993 / *Pst*I
P1_ANR	cgggatccCGTGGAATTGCTCACCTGC	4088940 / *BamH*I
P2_ANR	**gtttcagctCTCGGACAT**CACTTAATTCC	4089378
P3_ANR	**atgtccgagagctgaaaC**GTGCGATCTTTGG	4090208
P4_ANR	aactgcagGCAGGTCGATAATCGCAGC	4090722 / *Pst*I

Promoter-*lacZ* fusion constructs		Acc. # AJ548826
sylAp_f	ggaaTTCACATTGAGACACACATG	3062 / *Eco*RI
sylAp_r	aactgcaGCACTCTGTTCGAACTCC	3592 / *Pst*I
sylDp_f	ggaaTTCTCCATCGGCGGAC	9456 / *Eco*RI
sylDp_r	aactgcAGACATGACCATCTCCAC	9736 / *Pst*I
sylEp_f	ggaATTCGGTATGCGCTATGAC	22039 / *Eco*RI
sylEp_r	aactgCAGACATATGTAGACCTC	22370 / *Pst*I
sylBp_-226_f	ggaaTTCATGACGGCCTCGGA	5650 / *Eco*RI
sylBp_-153_f	ggaattcTTAGCGGTGATTGACTTC	- / *Eco*RI
sylBp_-117_f	ggaattCACCGCCAACTGTCTACC	- / *Eco*RI
sylBp_-105_f	ggaatTCTACCTGTAAAAAATAGCAG	- / *Eco*RI
sylBp_-90_f	ggaattcTAGCA GCTTACGCGGCTC	- / *Eco*RI
sylBp_-75_f	ggaattCTCCCCGCCCCTCTC	- / *Eco*RI
sylBp_-67_f	ggaattCCCTCTCTAGTCTATATAAC	- / *Eco*RI
sylBp_-57_f	ggaatTCTATATAACGCCCTAATTAC	- / *Eco*RI
sylBp_r	aactgcagGTTGGACGGTCTGCAT	5409 / *Pst*I
sylCp_-239_f	ggaatTCGTTGGACGGTCTGC	5407 / *Eco*RI
sylCp_-175_f	ggaattCGTTATATAGACTAGAGAG	- / *Eco*RI
sylCp_-124_f	ggaattCAGGTAGACAGTTGGC	- / *Eco*RI
sylCp_-110_f	ggaattcGCGGTGGCGTGTGGTC	- / *Eco*RI
sylCp_-96_f	ggaatTCTTTTTAAAGAAGTCAATC	- / *Eco*RI
sylCp_-82_f	ggaatTCAATCACCGCTAACAAAAACG	- / *Eco*RI
sylCp_-68_f	ggaattCAAAAACGGATACACCG	- / *Eco*RI
sylCp_r	aactgcaGTTCATGACGGCCTCGGAT	5651 / *Pst*I

Probes for MDAE		Acc. # AJ548826
sylAp_r_biot	GCACTCTGTTCGAACTCC	3592 /-
sylBp_r_biot	GTTGGACGGTCTGCATC	5409 / for sylBp deletions
sylCp_r_biot	TTCATGACGGCCTCGGAT	5650 / for 244 bp frag
sylCp_-155_f	GGCGGGGAGCCGCGTAAG	for 64 bp fragment
sylCp_-92_r_biot	AAAGACCACACGCCACCG	for 64 bp fragment
sylC_r_biot	TAGCCGAGTGACAACGAGG	7684 / negative control

sylC_N_f	GGTATTCTGCGACGGTGTTT	7565 / negative control
Constructs for MBP-fusion proteins		
sylAFP_f	cgggatccTGGAGTAGAGTGATGGC	3495 / *BamH*I / AJ548826
sylAFP_r	cccaagcTT CCG ATCGGCCTC G	4336 / *Hind* III / AJ548826
salAFP_f	ccggaattcCAGCTTTTCCCG CATC	7987 / *EcoR*I / AF372703
salAFP_r	cccaagctTGAACAGGGTGGTCGT T	9007 / Hind III / AF372703
Overexpression constructs		Acc. # AJ548826
salA_OE_f	ccatcgatAAAGGAACCCCGATGAAC	7963 / *Cla*I
salA_OE_r	ggaaTTCTGCTCAGACAGCTGCCTG	8835 / *EcoR*I
LacZp_OL_sylAp	**gtcaccaagAATTAATGCA**GCTGG	
LacZp_OL_sylBp	**aggccgtcatATTAATGCAG**CTGG	
LacZp_OL_sylCp	**accgtccaacATTAATGCAG**CTGG	
sylAp_OL_lacZp	**tgcattaattcttggtgAC**ATTGAGACACATGAGG	3065
sylBp_OL_lacZp	**ctgcattaatATGACGGCCT**CGGA	5647
sylCp_OL_lacZp	**ctgcattaatGTTGGACGGT**CTGC	5409

[a] Forward and reverse primers are labelled with _f and _r suffixes respectively. Numbers in the primer names for the sylB/sylC promoter deletion fragments correspond to the positions upstream of the sylB and sylC translation initiation codons, respectively

[b] Primer extensions that are not part of the genomic sequences are given in lower case letters, parts added for overlap extension PCR in bold letters.

[c] Primer positions refer to sequences available under the GeneBank accession numbers indicated. Restriction sites added for cloning are indicated.

4.

PSEUDOMONAS SYRINGAE VIRULENCE FACTOR SYRINGOLIN A COUNTERACTS STOMATAL IMMUNITY BY PROTEASOME INHIBITION

Barbara Schellenberg[1], Christina Ramel[1], and Robert Dudler[1]

MPMI 2010

Vol. 23, No. 10, pp. 1287–1293

Accepted 20 June 2010

[1] Institute of Plant Biology, University of Zurich, Zollikerstrasse 107, CH-8008 Zurich, Switzerland

4.1 Abstract

The peptide derivative syringolin A, a product of a mixed non-ribosomal peptide/polyketide synthetase, is secreted by certain strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* (*Pss*). Syringolin A was shown to be a virulence factor for *Pss* B728a because disease symptoms on its host *Phaseolus vulgaris* (bean) were greatly reduced upon inoculation with syringolin A-negative mutants. Syringolin A's mode of action was recently shown to be irreversible proteasome inhibition. Here we report that syringolin A-producing bacteria are able to open stomata and thus counteract stomatal innate immunity in bean and Arabidopsis. Syringolin A-negative mutants, which induce stomatal closure, can be complemented by exogenous addition of not only syringolin A, but also MG132, a well-characterized and structurally unrelated proteasome inhibitor. This demonstrates that proteasome activity is crucial for guard cell function. In Arabidopsis, stomatal immunity was salicylic acid (SA)-dependent and required NPR1, a key regulator of the SA-dependent defence pathway whose proteasome-dependent turnover has been reported to be essential for its function. Thus, elimination of NPR1 turnover through proteasome inhibition by syringolin A is an attractive hypothesis to explain the observed inhibition of stomatal immunity by syringolin A.

4.2 Introduction

As a defence against pathogens plants have evolved an innate immune system. As currently understood, it has two branches (Boller and Felix, 2009; Chisholm et al., 2006; Cui et al., 2009; Jones and Dangl, 2006). One branch concerns the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) located predominantly on the surface of host cells. Recognition triggers defence responses that collectively restrict pathogen growth, leading to basal, non-race-specific resistance dubbed PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). However, pathogens have evolved effectors able to suppress PTI. In bacterial pathogens, which are particularly well studied, many effector molecules are proteins injected into the host cytoplasm by the type III secretion system (T3SS) where they interfere with PTI signal transduction, thus leading to effector-triggered susceptibility (Guo et al., 2009; He et al., 2007; Mansfield, 2009; Metraux et al., 2009). As a second line of defence, plants have evolved effector recognition systems that trigger a strong but race-specific defence response (effector-triggered immunity; ETI) that often involves programmed cell death (Jones and Dangl, 2006).

To cause disease, plant pathogens have to invade host tissue. It has recently been reported that apart from wounds, stomata are entry ports for bacterial pathogens that are relevant for disease progression, and that stomatal closure upon pathogen attack is part of plant innate immunity (Liu et al., 2009; Melotto et al., 2008; Melotto et al., 2006). The importance of the stomatal innate immune response is also evident from the fact that bacterial effectors have been identified which counteract PAMP-induced stomatal closure. For example, the polyketide toxin coronatine, a virulence factor secreted by certain strains of *Pseudomonas syringae*, was shown to actively open stomata (Melotto et al., 2006). A similar activity was also reported of a factor of unknown identity secreted by *Xanthomonas campestris* (Gudesblat et al., 2009).

We have previously characterized syringolin A, a peptide derivative which is produced by a mixed non-ribosomal peptide synthetase (NRPS)/polyketide synthetase (PKS) and

secreted by some strains of *Pseudomonas syringae* pv. *syringae* (*Pss*) (Amrein et al., 2004; Imker et al., 2009; Ramel et al., 2009; Wäspi et al., 1998a; Wäspi et al., 1999). Recently, we reported syringolin A produced by *Pss* strain B728a to be a virulence factor because disease symptoms on its host *Phaseolus vulgaris* (bean) were greatly reduced upon inoculation with syringolin A-negative mutant as compared to wild-type bacteria (Groll et al., 2008). The cellular target of syringolin A was identified to be the proteasome. It was demonstrated that syringolin A irreversibly inhibited all three catalytic activities of the eukaryotic proteasome and forms, together with the glidobactins, a new structural class of proteasome inhibitors that act by a novel mechanism (Groll et al., 2008; Schellenberg et al., 2007).

Because the proteasome plays a central role in numerous regulatory pathways such as hormone signaling and responses to environmental stimuli and pathogens (Vierstra, 2009), inhibition of the proteasome is expected to elicit pleiotropic responses and it is not evident from which of these the pathogen would benefit. Here we report that syringolin A-producing bacteria are able to open stomata and thus counteract stomatal innate immunity in bean and Arabidopsis. It likely does so by its capacity as a proteasome inhibitor because the same effect was also observed by treatment with MG132, a well-characterized and structurally unrelated proteasome inhibitor. This suggests that proteasome function is crucial for guard cell function. Stomatal immunity in Arabidopsis was dependent on NPR1, an important regulator of salicylic acid (SA)-dependent defence pathways. Because NPR1 function requires its turnover by the proteasome (Spoel et al., 2009), suppression of NPR1 turnover by proteasome inhibition is an attractive hypothesis explaining the observed action of syringolin A on stomata.

4.3 Results

4.3.1 Syringolin A counteracts pathogen-induced stomatal closure in bean by proteasome inhibition

First we tested whether the increased number of disease symptoms resulting from infections of bean with the syringolin A-producing *Pss* strain B728a as compared to infection with syringolin A-negative mutant strains might be directly linked to *in planta* growth differences between the two genotypes. Thus, leaves of three-week-old bean plants (*Phaseolus vulgaris* cultivars 'Bush Blue Lake') were spray-inoculated with the wild-type strain B728a and the syringolin A-negative mutant derivative B728a KO_sylC (Groll et al., 2008). Endophytic bacterial growth was determined over five days. Whereas both the wild type and the mutant were able to grow endophytically, the mutant reached lower (within a factor of 10) population densities as compared to the wild type (Figure 1). In some experiments, as in the one shown in Figure 1, the mutant strain transiently seemed to catch up with the wild type but seemed to decline faster. However, in other experiments this was not obvious (Supplementary Figure S1). Similar patterns were observed in independent repetitions of the experiment, also in a second cultivar ('Winnetou'; data not shown). The most prominent difference was always observed 6 hours post inoculation (hpi), the earliest time point measured. This suggested that wild-type bacteria reach the endophytic leaf space more efficiently than syringolin A-negative mutant bacteria.

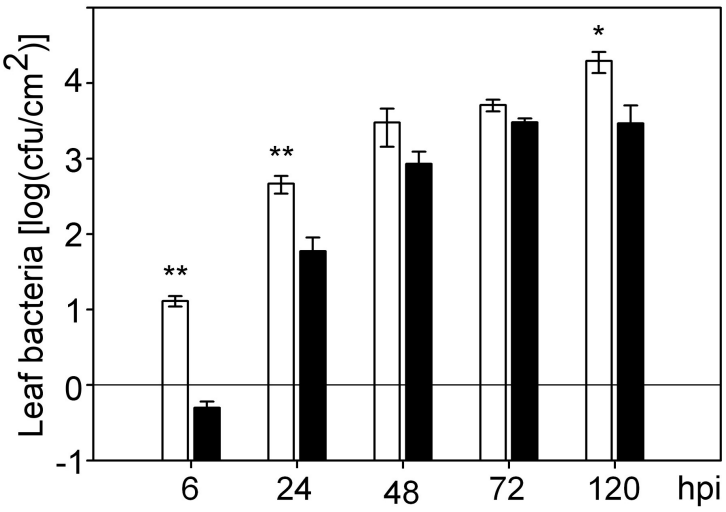


Figure 1. Endophytic growth of wild-type and syringolin A-negative *Pss* B728a strains on bean.
Leaves of *P. vulgaris* (cv. 'Bush Blue Lake') were spray-inoculated with suspensions (10^7 cfu/ml) of the wild-type *Pss* B728a strain (open bars) or the syringolin A-negative mutant *Pss* B728a KO_sylC (black bars). The means ± standard error of 5 plants per treatment are given (n=5). Asterisks indicate significant differences between the strains (Student's *t*-test; ** $P<0.01$; * $P<0.05$). The experiment was independently repeated once using cv. 'Bush Blue Lake' and 3 times using cv. 'Winnetou' with similar results.

Because bean leaves were spray-inoculated without damaging tissue, plant stomata likely represented the only entry point into the inner leaf tissue. We therefore investigated whether syringolin A would affect stomatal aperture. Abaxial epidermal peels were prepared from primary leaves of two-week-old bean plants (cv. 'Bush Blue Lake' and 'Winnetou') kept in the light prior to the experiment (stomata are open) and incubated in the light in control solution or bacterial suspensions of the syringolin A-producing wild-type strain *Pss* B728a and the syringolin A-negative knock-out mutant of this strain (B728a KO_sylC). One hour after incubation, epidermal peels were mounted on glass slides in high humidity and apertures of all stomata present on photomicrographs were measured. After three hours of incubation under the same conditions, stomatal aperture was measured again on photomicrographs taken from the same slides. The results are depicted in Figure 2A and show that after 3 h of incubation with the wild-type strain, epidermal peels exhibited open stomata with an aperture comparable to the control without bacteria, whereas the syringolin

A-negative mutant led to significantly reduced stomatal apertures. Thus, syringolin A-producing bacteria were able to keep open or reopen stomata.

We next tested whether exogenous addition of isolated syringolin A could revert stomatal closure induced by syringolin A-negative bacteria. Epidermal peels of light-exposed bean plants were incubated with mutant bacteria for one hour before syringolin A was added to a final concentration of 20 µM. As evident from Figure 2B, two hours after syringolin A addition, stomata openings were significantly wider than on the control-treated peels. The same result was obtained when syringolin A was substituted by MG132, a well established, structurally unrelated, reversible proteasome inhibitor (Figure 2B). These results suggest that syringolin A in its capacity as a proteasome inhibitor was responsible for the (re)opening of stomata observed with wild-type bacteria.

To clarify whether syringolin A-synthesizing bacteria were capable to actively open stomata, bean plants were pre-incubated in the dark, where stomata are normally closed. Epidermal peels prepared from these plants were then incubated in the dark with suspensions of wild-type and syringolin A-negative mutant bacteria or in control solution. One hour after incubation, all epidermal peels showed closed stomata, while after three hours, stomata on peels incubated with wild-type bacteria were significantly more open when compared to the ones incubated with suspensions of syringolin A-negative bacteria or with control solution (Figure 2C). In a further experiment, epidermis peels of bean plants kept in the dark were incubated in the dark in the presence of 20 µM syringolin A or 100 µM MG132. As shown in Figure 2D, both proteasome inhibitors caused closed stomata to open, suggesting that proteasome activity is crucial for guard cell function in bean.

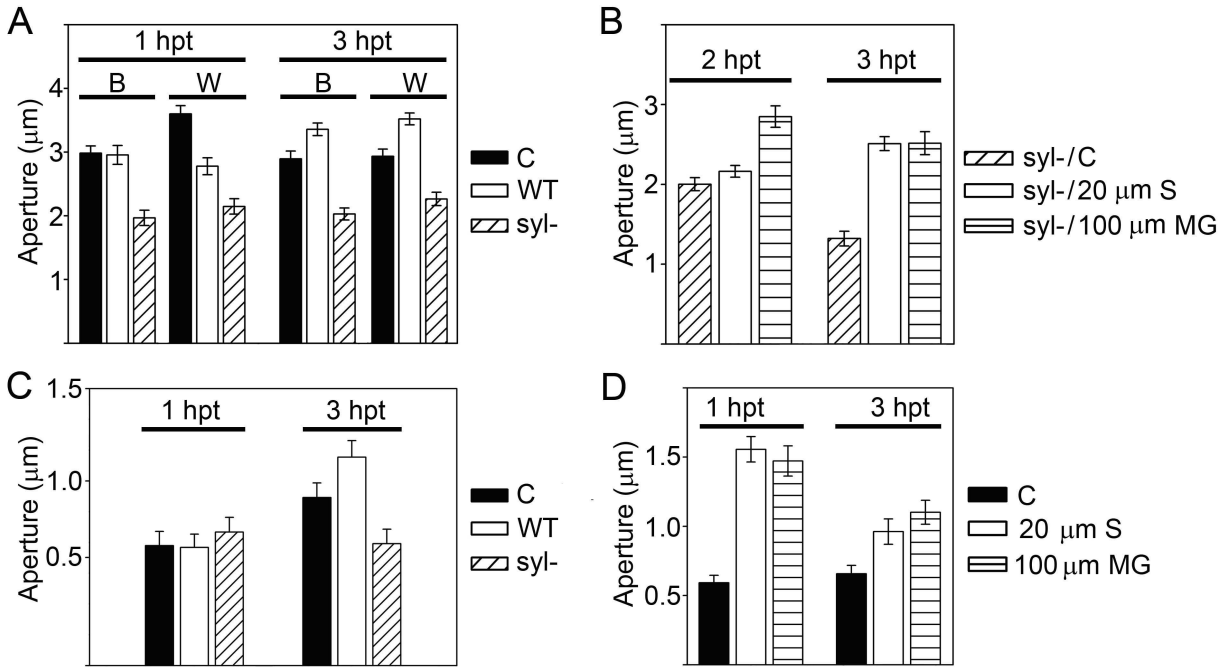


Figure 2. **Pathogen-induced stomatal closure is counteracted by syringolin A in bean.**

A, Apertures of stomata on epidermal peels of bean cultivars 'Bush Blue Lake' (B) and 'Winnetou' (W) 1 h and 3 h after treatment with control buffer, wild-type, or syringolin A-negative *Pss* B728a strains.

B, Complementation of syringolin A-negative *Pss* B728a by exogenous addition of syringolin A or MG132. Epidermal peels of bean cultivar 'Winnetou' were preincubated with bacteria for 1 h before addition of the chemicals. Stomatal apertures were measured after another 1 and 2 h.

C, Stomatal apertures of epidermal peels from bean leaves (cv. 'Winnetou') kept in the dark were incubated in the dark with control buffer, wild-type (WT), and syringolin A-negative *Pss* B728a bacteria.

D, Stomatal apertures of epidermal peels from bean leaves (cv. 'Winnetou') kept in the dark were incubated in the dark with control buffer, syringolin A, and MG132.

A-D, Means ± standard errors are given (n ≥ 70 for each data point). WT, wild type; syl-, syringolin A-negative mutant; hpt, hours post treatment. Experiments were independently performed twice (C) or 3 times (A, B and D). C, control buffer; S, syringolin A; MG, MG132. Asterisks denote significant differences (Student's *t*-test; ** $P<0.01$) between WT and syl- strains (**A, C**), or between treatment with syringolin A or MG132 and the control treatment (**B, D**).

Of the many plant hormones that affect guard cell function, stomatal closure by abscisic acid (ABA) plays a particularly crucial role (for a review see (Acharya and Assmann, 2009). Therefore, we wanted to test whether syringolin A and MG132 counteract ABA-induced stomatal closure in bean. Epidermal peals of bean plants (cv. Winnetou) kept in the

light were incubated in the light in presence of 30 µM ABA and increasing concentrations of syringolin A or MG132. As evident from Figure 3, both proteasome inhibitors partially counteracted ABA-induced stomatal closure in a concentration-dependent manner.

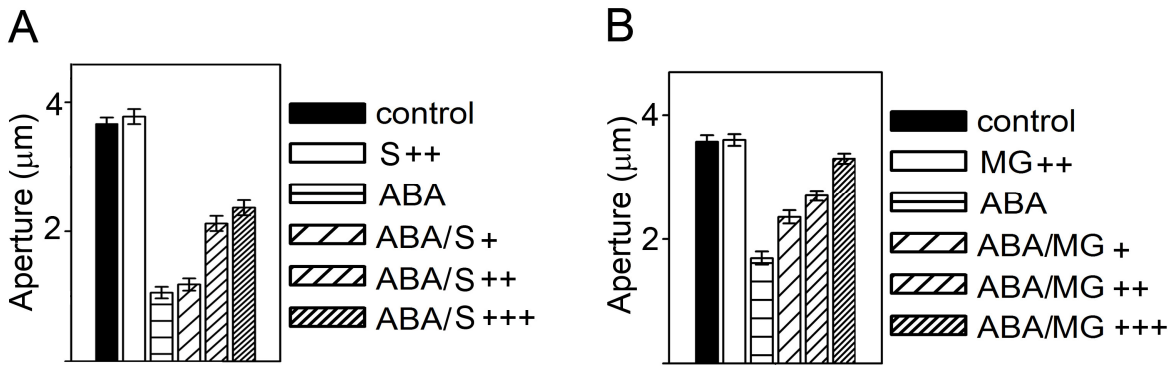


Figure 3. **Interference of proteasome inhibitors with ABA-induced stomatal closure**.
Stomatal apertures (means ± standard errors; n ≥ 70 for each data point) on epidermal peels of bean leaves (cv. 'Winnetou') 3 h after treatment with ABA and syringolin A **(A)** or ABA and MG132 **(B)** . Control, control solution; ABA, 30 µM abscisic acid. S+, S++, S+++; 10, 20, and 40 µM syringolin A, respectively. MG+, MG++, MG+++; 50, 100, and 200 µM MG132, respectively. Asterisks indicate significant differences (Student's *t*-test; ** $P<0.01$) between treatment with syringolin A or MG132 and treatment with ABA alone. Stomatal apertures after ABA treatment are also significantly smaller ($P<0.01$) than after control treatment or treatment with syringolin A or MG132 alone.

4.3.2 Proteasome function is also crucial for the stomatal immune response in Arabidopsis

Melotto and co-workers (Melotto et al., 2008; Melotto et al., 2006) reported stomatal closure to be part of PTI in Arabidopsis towards bacterial pathogens like *P. syringae* pv. *tomato* (*Pst*) DC3000 or PAMPS, which was counteracted by the toxin coronatine produced by *Pst* DC3000. Therefore, we explored whether the proteasome played a role in the stomatal immune response in Arabidopsis using the coronatine-negative mutant *Pst* DC3118 (Métraux et al., 1991) in experiments with Arabidopsis (accession Col-0) epidermal peels. As shown in

Figure 4, the mutant, but not the wild type, led to stomatal closure as reported in the literature (Melotto et al., 2006).

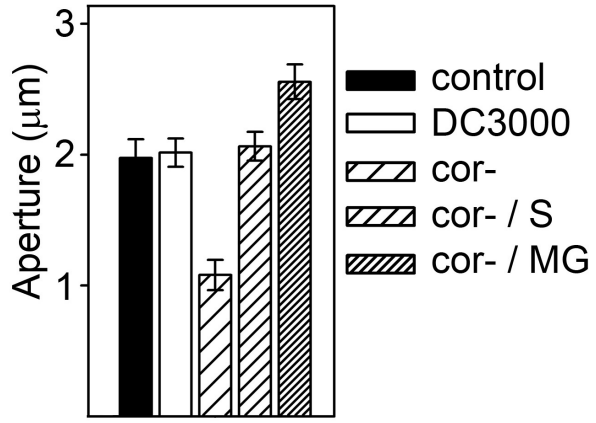


Figure 4. Syringolin A and MG132 reverse PAMP/pathogen-induced stomatal closure in Arabidopsis.
Epidermal peels of Arabidopsis (Col-0) were incubated with the *Pst* DC3000 wild-type strain (DC3000) or coronatine-negative strain *Pst* DC3118 (cor-) with and without 20 µM syringolin A (S) or 100 µM MG132 (MG). Means ± standard errors of stomatal apertures (n ≥ 70 for each data point) 3 hours after treatment (hpt) are given. The experiment was independently repeated once with similar results. Asterisks indicate significant differences (Student's *t*-test; ** $P<0.01$) between treatment with the cor- strain and treatments with the cor- strain and syringolin A or MG132, respectively.

This stomatal immune response was counteracted by exogenous addition of solutions containing 20 µM syringolin A or 100 µM MG132, suggesting that proteasome function was necessary for PTI-associated stomatal immunity in Arabidopsis. Treatment of Arabidopsis (Col-0) epidermal peels with wild-type and syringolin A-negative mutant *Pss* B728a strains, for which Arabidopsis is not a host, led to open and closed stomata, respectively, as was observed in bean (data not shown). Similar results as in bean were also obtained with dark-incubated Arabidopsis epidermal peels in experiments analogous to the ones shown in Figure 2C and 2D (data not shown).

Using mutants and pharmacological agents, Melotto and co-workers showed that in Arabidopsis, pathogen/PAMP-induced stomatal closure was dependent on the salicylic acid (SA)-regulated defence pathway and on abscisic acid (ABA) (Melotto et al., 2008; Melotto et

al., 2006). The proteasome was reported to play a role in the regulation of NPR1, a key regulator in the SA-dependent defence pathway (Spoel et al., 2009). The importance of proteasome activity for NPR1 function was corroborated by the observation that (1) syringolin A greatly reduced SA-induced *PATHOGENESIS-RELATED PROTEIN 1* (*PR-1*) transcript accumulation in spray application experiments with Arabidopsis, and that (2) infiltration of Arabidopsis with wild-type *Pss* B728a resulted in significantly reduced *PR-1* transcript accumulation as compared to infiltration with syringolin A-negative mutant bacteria (Supplementary Figure S2). In addition to the SA pathway, in Arabidopsis the proteasome was reported to be involved in the ABA signal transduction pathway, where it was proposed to control abscisic acid sensitivity by regulating nuclear localization of the abscisic acid-insensitive 1 (ABI1) protein (Moes et al., 2008). Proteasome inhibition by MG132 led to a marked increase of ABI1-mediated inhibition of ABA signaling (Moes et al., 2008). Thus, we explored whether these proteins were involved in the stomatal immune response to *Pss* B728a in Arabidopsis.

First we tested if the SA defence pathway was also necessary for the stomatal immune response triggered by the non-host pathogen *Pss* B728a, as reported for *Pst* DC3000 (Melotto et al., 2006). Indeed stomata did not close on epidermal peels from SA-deficient *nahG* transgenic Arabidopsis plants (Delaney et al., 1994) upon incubation with syringolin A-negative B728a KO_sylC mutant bacteria (Figure 5A), confirming the importance of the SA defence pathway. We then tested the stomatal immune response in *npr1* mutant plants (Cao et al., 1994). As evident from Figure 5B, *npr1* plants were unable to close stomata upon treatment with syringolin A-negative bacteria, indicating that NPR1 was necessary for the stomatal immune response. Thus, we consider inhibition by syringolin A of proteasome-mediated turnover of NPR1, which was shown to be essential for NPR1 function (Spoel et al., 2009), to provide an attractive hypothesis explaining the observed syringolin A-mediated inhibition of *Pss* B728a-induced stomatal closure.

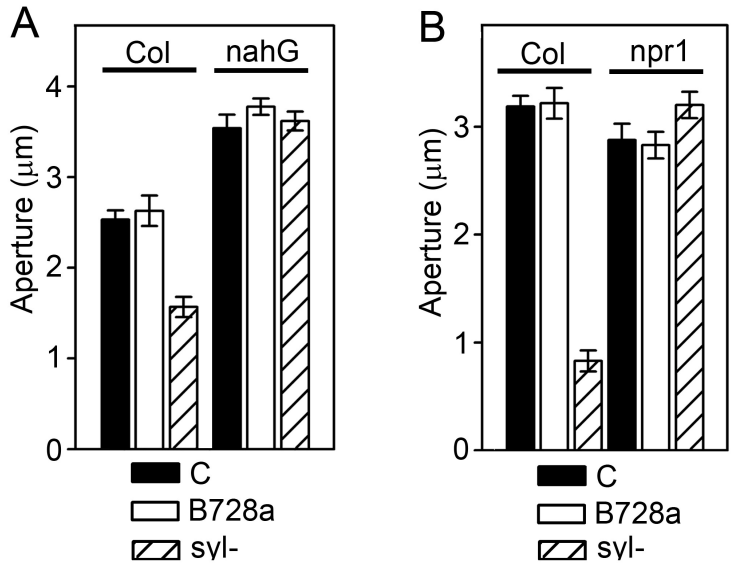


Figure 5. Stomatal response of Arabidopsis mutants affected in the SA signaling pathway.
Epidermal peels from Col-0 wild-type (Col) and *nahG* **(A)** or *npr1* **(B)** plants were treated with control solution (C), *Pss* B728a and syringolin A-negative *Pss* B728a KO_sylC (syl-) bacteria. Means ± standard errors of stomatal apertures (n ≥ 70 for each data point) 3 hours after treatment (hpt) are given. The experiments were independently performed twice (A) and three times (B) with similar results. Asterisks denote significant differences (Student's *t*-test; ** $P<0.01$) between treatment with wild-type and syringolin A-negative mutant bacteria in Col-0 plants.

Next, we explored whether pathogen-induced stomatal closure in Arabidopsis required the ABA signaling pathway in which ABI1 is involved. ABI1 belongs to the group-A protein phosphatase 2C (PP2C) family of proteins which negatively regulate ABA-signaling. They have recently been shown to be sequestered in a complex with the PYR/PYL/RCAR family of ABA receptor proteins in the presence of ABA, leading to their inactivation and the consequent de-repression of ABA responses (Fujii et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009a; Santiago et al., 2009b). Because functional redundancy within the PP2C family usually does not result in a mutant phenotype of individual loss-of-function PP2C mutants, we made use of the dominant ABA-insensitive *abi1-1* allele (Koornneef et al., 1984). Epidermal peels from both wild-type and *abi1-1* Arabidopsis plants reacted to syringolin A-negative mutant *Pss* B728a by closing their stomata (Figure 6A), indicating that the PP2C/PYL/PYR/RCAR-mediated ABA signaling

pathway was not essential for the stomatal response to this non-host bacterium. This was in contrast to the stomatal response elicited by coronatine-negative *Pst* DC3118, which required this pathway as stomata on epidermal peels of *abi1-1* plants were unable to close upon treatment with *Pst* DC3118 bacteria (Figure 6B). The control experiment shown in Figure 6C showed that, as expected, wild-type, but not *abi1-1* plants, responded with stomatal closure to exogenous addition of ABA.

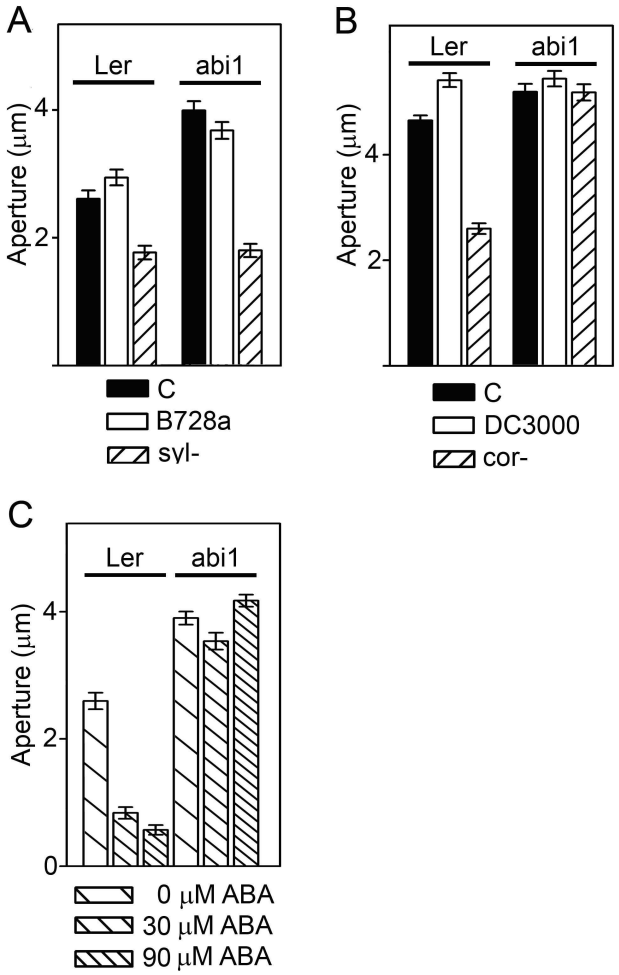


Figure 6. Stomatal immune response in the *abi1-1* Arabidopsis ABA-signaling mutant.

Epidermal peels from L*er* wild-type and *abi1-1* mutant Arabidopsis plants were treated with control solution (C), *Pss* B728a (B728a), and syringolin A-negative *Pss* B728a KO_sylC (syl-) bacteria **(A)**, or with *Pst* DC3000 (DC3000) and coronatine-negative *Pst* DC3118 (cor-) bacteria **(B)**, or with the indicated concentrations of ABA **(C)**. Means ± standard errors of stomatal apertures (n ≥ 70 for each data point) 3 hours after treatment (hpt) are given. The experiments were independently performed twice (A, C) and three times (B) with similar results. Asterisks denote significant differences (Student's *t*-test; ** $P<0.01$) between treatment with wild-type and syringolin A-negative (**A**) and coronatine-negative (**B**) bacteria, respectively, or between control treatment and ABA treatment (**C**).

4.4 Discussion

Spray-inoculation of bean plants with wild-type and syringolin A-negative mutant *Pss* B728a bacteria revealed that both strains were able to multiply endophytically, although the wild type reached moderately higher population densities than the mutant. Apparently, once inside the leaf, both bacterial genotypes are able to multiply at similar rates, although persistence may be somewhat lowered in the mutant. The facilitated entry that syringolin A-producing bacteria experience may well be relevant for disease etiology, at least under certain environmental conditions. Our results are compatible with earlier observations reported in the literature. Willis et al. (1990) infiltrated the B728a strain and the *lemA* (later renamed *gacS*) mutant NPS3136 into bean leaves where both strains were able to multiply to similar extents, although the mutant was deficient in lesion formation. The *lemA* mutant has a non-functional *gacS/gacA* global regulatory two-component system which also controls syringolin A biosynthesis (Reimmann et al., 1995; Wäspi et al., 1998b). Spray-inoculation of bean plants with these strains in field experiments revealed that the *lemA* gene contributed to the field fitness as the mutant strain reached lower population densities associated with bean leaves (Hirano et al., 1997). Although in these experiments epiphytic and endophytic growth were not distinguished, and although the *gacS/gacA* regulon certainly affects other properties of the bacterium in addition to syringolin A production, facilitated entry into leaves may have contributed to the reported observations.

The fact that the proteasome inhibitors syringolin A and MG132 are able to suppress stomatal immunity implied proteasome function to be essential for the stomatal immune reaction upon pathogen perception. The proteasomal degradation pathway has been implicated in at least some aspects of the signaling pathways identified by Melotto et al. (2006) to be important for pathogen/PAMP-induced stomatal closure. The proteasome plays a pivotal role in the SA-mediated transcriptional defence response. The key regulator NPR1, a transcriptional co-activator, must be turned over in its phosphorylated form by the proteasome to activate target genes involved in systemic acquired resistance (Spoel et al.,

2009). We have shown that the stomatal immune response to *Pss* B728a is impaired in the *npr1* mutant, indicating that NPR1 function is necessary for this response. Thus, inhibition of proteasomal NPR1 turnover by syringolin A provides a likely explanation for the observed syringolin A-mediated inhibition of stomatal closure.

With regard to ABA signaling, PP2Cs like ABI1 and homologues must be located in the nucleus to bind and inhibit their target kinases, as e.g. OST1 (Vlad et al., 2009). Nuclear location was at least in part regulated by the proteasome because MG132 led to preferential localization of wild-type ABI1 mutant abi1-1 proteins in the nucleus and to enhanced inhibition of ABA signaling (Moes et al., 2008). Thus, inhibition of *Pst* DC3118-triggered PTI-associated stomatal closure in Arabidopsis by exogenous syringolin A and MG132 (Figure 4) could also be explained by interference with the PYR/PYL/RCAR-PP2C-mediated ABA signaling pathway which is required for the response to this pathogen (Figure 6B). This may also apply to the *Pss*/bean pathosystem provided that the wiring of the PTI-associated stomatal immune response in this pathosystem is similar to the one in the *Pst*/Arabidopsis system, which is currently not known. In contrast, the stomatal immune response triggered in Arabidopsis by the non-host pathogen *Pss* B728a was not dependent on the PPC2-mediated ABA signal transduction pathway (Figure 6A).

The stomata are entry points into plants for many pathogens. It is thus not surprising that the regulation of stomatal aperture upon pathogen perception is part of the plant immune response (Liu et al., 2009; Melotto et al., 2008; Melotto et al., 2006). On the other hand, it is increasingly becoming clear that pathogens have evolved diverse means to counteract stomatal immunity. The most widely known agent is the wilting toxin fusicoccin produced by *Fusicoccum amygdali*, a pathogen of almond and peach trees (Ballio et al., 1964; Marre, 1979). It leads to stomatal opening by activating the plasma membrane H^+-ATPase in complex with a 14-3-3 protein (Baunsgaard et al., 1998; Olivari et al., 1998). Other agents that overcome stomatal immunity in Arabidopsis include a recently described small diffusible factor of unknown identity from *Xanthomonas campestris* (Gudesblat et al., 2009) and the

already mentioned toxin coronatine which is secreted by certain *P. syringae* strains like *Pst* DC3000 (Melotto et al., 2006).

Coronatine is a mimic of isoleucine-conjugated jasmonic acid (JA) and activates the JA signaling pathway in a *CORONATINE INSENSITIVE-1* (*COI1*) dependent manner. COI1 is an F-box protein and part of the SCF^{COI1} E3 ubiquitin ligase that targets repressors of JA responses to proteasomal destruction (Chini et al., 2007; Thines et al., 2007). Because syringolin A is a proteasome inhibitor and coronatine needs host proteasome function for its action, syringolin A and coronatine seem to be incompatible if produced by the same pathogen at the same time. Indeed, none of the limited number of strains whose genome sequence is known or that we checked by PCR-based methods contains both syringolin A synthetase genes and genes for coronatine biosynthesis. Syringolin A production would seem also to be incompatible with the concurrent expression of effectors like HopAB2 (formerly named AvrPtoB), which contains E3 ligase activity and targets host defence proteins to proteasomal destruction (Rosebrock et al., 2007). While the *Pss* B728a genome, in contrast to the one of *Pst* DC3000, does not contain a *hopAB2* gene (Sarkar et al., 2006), it is currently not known whether strains exist capable of expressing syringolin A and effectors requiring host proteasome activity for their action. If such strains exist, interference may be avoided by exclusive expression of one or the other depending on time and conditions.

4.5 Material and methods

Plant material, bacterial strains, and growth conditions

Phaseolus vulgaris (bean) plants (cultivars ‘Bush Blue Lake 274’ and ‘Winnetou’) were cultivated with a 16 h light/8 h dark regime at a mean air temperature of 21° C and a humidity of 50-60 %. Arabidopsis seeds were surface-sterilized with a solution containing 1 % (v/v) sodium hypochlorite and 0.03 % (v/v) Triton X-100 for 15 min, washed three times with sterile

water and stratified for 3 to 4 days at 4° C in the dark. Seeds were aligned on half-strength Murashige and Skoog (MS) plates containing 2 % (w/v) sucrose and 0.6 % phytagel (Sigma-Aldrich, Buchs, Switzerland). Plates were placed in vertical orientation in an incubator under long-day conditions (16 h light/8 h dark) and 21° C for approximately 10 days. Seedlings were then transferred into soil and kept under the same conditions.

Construction of the syringolin A-negative *Pss* mutant B728a KO_sylC by plasmid insertion into the *sylC* gene of the syringolin A synthetase gene cluster has been described (Amrein et al., 2004; Groll et al., 2008). All strains were grown at 28° C on Luria-Bertani (LB) plates [10 g/l bactotryptone (Difco, Chemie Brunschwig AG, Basel, Switzerland); 5 g/l bactoyeast extract (Difco); 10 g/l NaCl; 15 g/l agar (Difco)] supplemented with 50 µg/ml rifampicin (Rif) for wild-type and 50 µg/ml Rif, 10 µg/ml tetracycline (Tet) for mutant bacteria.

Inoculation of bean plants and determination of endophytic bacterial growth

For spray-inoculation of bean plants, bacterial inocula were essentially prepared as described (Hirano et al., 1997). In short, bacterial over-night cultures grown in LB without antibiotics were diluted to an OD_{600} of 1.0 (about $5x10^8$ colony forming units per ml [cfu/ml]). One hundred µl of this dilution were plated onto LB plates (9 cm diameter) and incubated for 2 days at 28° C. Bacteria were scraped off the plates with a spatula, resuspended in distilled water and diluted to 10^7 cfu/ml. The bacterial suspensions were sprayed onto both sides of primary leaves of two-week-old bean plants which had been incubated in a climate chamber at 23° C and a light intensity of 17 µE for 2 h prior to inoculation. The spray-inoculated plants were covered with a clear, light-permeable plastic lid for 24 h to maintain high humidity.

For each treatment and time point, endophytic bacterial growth was determined from one leaf of five different plants. Per leaf, 3 samples of 6 discs with a diameter of 1 cm were excised. Each sample was surface-sterilized in 15% (v/v) H_2O_2 for 5 min, washed in sterile water and homogenized in 1 ml 10 mM $MgSO_4 \bullet 7H_2O$ with a plastic pestle in an Eppendorf vial. The three extracts were pooled and diluted stepwise by a factor of 10 in 10 mM $MgSO_4 \bullet 7H_2O$. Six twenty-µl droplets of each dilution were placed onto antibiotic-free LB

plates. The plates were incubated at 28° C for 20 hours. Bacterial colonies were counted, averaged over the replicate platings of the same dilution and normalized to cfu/cm^2 leaf area.

Preparation and treatment of epidermal peels and determination of stomatal apertures

To determine stomatal apertures, bean and Arabidopsis plants were incubated in a climate chamber under light (130 µE) or in the dark at 23° C for at least 3 h prior to use. Bacterial over-night cultures grown in LB medium were washed and diluted in distilled water to $5x10^8$ cfu/ml. Silwet L-77 was added to a final concentration of 0.025%. Distilled water containing 0.025% Silwet L-77 was used as the control solution and also used to prepare solutions of chemicals. Bacterial suspensions and solutions of chemicals were dropped onto a glass slide bordered with Scotch tape, and epidermal peels prepared from the abaxial leaf side were directly mounted on the liquid layer and covered with the corresponding suspensions/solutions and a cover slide. To prevent desiccation, glass slides were put into a tray lined with wet household paper and incubated in a climate chamber at 23° C.

In mutant complementation experiments, epidermal peels were incubated in small petri dishes containing bacterial suspensions of $5x10^8$ cfu/ml for one hour to trigger the basal resistance reaction. The peels were then transferred onto a glass slide carrying a liquid layer with the desired chemical solution, covered with the respective solution and a cover slide and incubated as described above. The preparations were incubated under the same light conditions as the whole plants were prior to their use. One and 3 hours after treatment (hpt), or 2 and 3 hpt in case of mutant complementation experiments, digital pictures of epidermal peels were taken with a Leica DFC420 camera (Leica Microsystems, Wetzlar, Germany) mounted on a Laborlux S microscope (Leitz, Wetzlar, Germany) equipped with a 20x objective . Stomatal apertures were measured on photographs using ImageJ version 1.4.1 software (Abramoff, 2004). Apertures of all stomata in focus on a digital image were measured. Every data point represented the average from 70 to 200 stomata from a total of 10 non-overlapping images taken.

Syringolin A was prepared as described (Amrein et al., 2004; Wäspi et al., 2001) and MG132 was purchased from Biomol (ANAWA Trading SA, Wangen Zürich, Switzerland). All other chemicals were obtained from Sigma-Aldrich (Buchs, Switzerland).

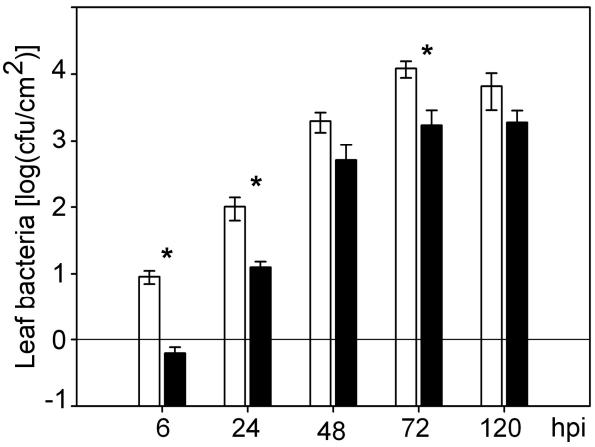
RNA gel blot analysis of chemically treated and infiltrated Arabidopsis plants

Three-week-old Arabidopsis (Col-0) plants were sprayed with 0.05 % Tween 20 solutions containing 2.5 mM salicylic acid supplemented with or without 50 µM syringolin A until droplets started to role off the leaves. Alternatively, leaves were infiltrated with suspensions containing 10^6 colony-forming units (cfu) per milliliter distilled water of wild-type *Pss* B728a or syringolin A-negative *Pss* B728a KO_sylC bacteria. Total RNA was extracted 18 h after treatment and subjected to gel blot analysis with a ^{32}P-labelled *PR-1* cDNA hybridization probe (Uknes et al., 1992) using standard procedures (Ausubel et al., 1987).

Acknowledgments

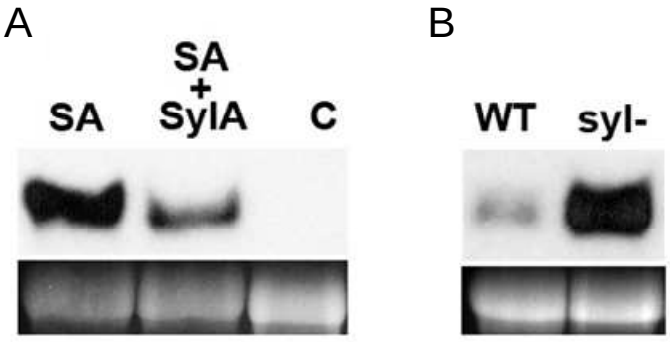
We thank Stefan Hörtensteiner, Felix Mauch, Brigitte Mauch-Mani, and Jean-Pierre Métraux for Arabidopsis and *Pseudomonas* mutants, and Stefan Hörtensteiner for comments on the manuscript. Financial support from the Swiss National Science Foundation (grant 3100A0-115970) and the University of Zurich is gratefully acknowledged.

Supplementary figures



Supplementary Figure S1. Endophytic growth of wild-type and syringolin A-negative *Pss* B728a strains on bean.

Leaves of *P. vulgaris* (cv. 'Bush Blue Lake') were spray-inoculated with suspensions (10^7 cfu/ml) of the wild-type *Pss* B728a strain (open bars) or the syringolin A-negative mutant *Pss* B728a KO_sylC (black bars). The means ± standard error of 5 plants per treatment are given (n=5). Asterisks indicate significant differences between the strains (Student's *t*-test; * *P*<0.05). This experiment is an independent repetition of the one shown in Figure 1.



Supplementary Figure. S2. Reduction of *PR1* transcript accumulation by syringolin A.

A. Three-week-old Arabidopsis (Col-0) plants were sprayed with 2.5 mM salicylic acid (SA), 2.5 mM SA and 50 µM syringolin A (SA+SylA), or control buffer (C) containing 0.05 % Tween 20.

B. Arabidopsis leaves were infiltrated with 10^6 cfu/ml wild-type (WT) *Pss* B728a or syringolin-negative (syl-) *Pss* B728a KO_sylC bacteria. **A** and **B.** RNA was extracted 18 h after treatment, blotted, and hybridized to a ^{32}P-radiolabeled *PR-1* cDNA probe. Top panels: autoradiograms; bottom panels: photographs of ethidium bromide-stained gels before blotting.

5.

GENERAL DISCUSSION

In chapter 2 we have demonstrated that the *syl*-gene cluster is sufficient to direct the biosynthesis of *bona fide* syringolin A in the heterologous organisms *Pss* SM and *P. putida*. Both organisms do not produce syringolin A and do not contain a *syl*-cluster homologue in their genome. *Pss* SM is a strain originally isolated from wheat and *P. putida* is a nonpathogenic saprotrophic soil bacterium. The heterologous expression demonstrated that no other syringolin A-specific genes are necessary for syringolin A biosynthesis.

Although the *syl*-promoters translationally fused to the *lacZ* gene were shown to be active in *E. coli*, no syringolin A biosynthesis could be observed upon heterologous expression of the *syl*-gene cluster in *E.coli* (unpublished data). This is due to the fact that for the activation of NRPS/PKS complexes, the transfer of 4'-phosphopantetheine from coenzyme A to conserved serine residues in the sequences of PCP/ACP domains is essential, but *E. coli* is lacking the necessary 4'-phosphopantetheine transferase genes (Lambalot et al., 1996). To ensure the production of fully active holoenzymes and thereby facilitate syringolin A biosynthesis in *E. coli*, a strain with a stable chromosomal integration of the 4′-phosphopantetheine transferase gene *sfp* should be used (Gruenewald et al., 2004).

In vivo labelling of syringolin A with $NaH^{13}CO_3$ demonstrated that the special ureido linkage of the two valine residues is formed by the incorporation of hydrogen carbonate or carbon dioxide. Our hypothesis that the ureido bond formation is achieved by carbamylation of valine, mediated by the *sylC* gene product alone, is by now confirmed by *in vitro* experiments (Imker et al., 2009). Because the ureido linkage between the two valine residues is formed *in vitro* by isolated SylC protein alone, Imker et al. (2009) hypothesised that two valines are iteratively activated by a single SylC module comprising a single C-, A,- and PCP-domain, and that the ureido linkage is achieved by incorporation of bicarbonate/CO_2 by cyclisation of an initial N- carboxyaminoacyl-S-phosphopantetheinyl

enzyme intermediate (Imker et al., 2009). We would favour the hypothesis that the syringolin A synthetase complex contains two SylC modules, which both would activate a valine and link them via the ureido group. The reason is that in the gene cluster encoding the enzyme responsible for the biosynthesis of anabaenopeptin, which contains an arginine (or tyrosine) residue linked by an ureido group to a lysine residue, the modules activating the arginine (or tyrosine) and lysine residues are encoded next to each other (Christiansen et al., 2011). This suggests that the two modules can activate their cognate amino acids and link them via an ureido group, which leads to chain reversal. This scenario would imply differential translation efficiency of the polycistronic transcripts *sylC* and *sylD*, leading to two SylC modules per SylD polypeptide.

The side chain of syringolin A with the two valines and the ureido group is essential for the stabilisation of syringolin A in the binding pockets of the catalytic subunits of the proteasome (Groll et al., 2008). Glidobactin, which contains a fatty acid side chain without a ureido group and a more flexible ring structure due to the absence of the second double bond in the ring structure, exhibited also different inhibition properties. Most intriguingly, glidobactin A does not inhibit the caspase-like activity of the proteasome (Groll et al., 2008).

Furthermore, the presence of the ureido group may influence syringolin A's uptake into the plant cell. Syringolin A was shown to enter plant cells very efficiently (Hassa et al., 2000), in contrast to glidobactin A, which is hardly taken up (unpublished observation). Conversely, glidobactin A is efficiently taken up by fungal and mammalian cells, whereas syringolin A is not (Coleman et al., 2006). How syringolin A uptake takes place in plants is still an open question, but the involvement of a peptide transporter is a likely hypothesis.

In chapter 3 we showed that the LuxR type transcription factor SylA binds to and directly activates the *sylB* and *sylC* promoters. Furthermore, we demonstrated that SalA is mediating syringolin A biosynthesis. In a *salA* mutant, syringolin A production was abolished and the *sylB* and *sylC* promoter activity decreased to basal activities whereas the *sylA* promoter activity was reduced to about 50%. Interestingly, an overexpression of *salA* led to a 2-3 fold increase of *sylA* promoter activity, indicating that SalA is a positive regulator of

syringolin A biosynthesis. Because we were not able to show a binding of MBP-SalA fusion proteins to the *sylA* promoter, we assumed that this activation occurs indirectly. We presented evidence that *syrG* and *syrF*, which are both part of the SalA regulon, are not involved in syringolin A biosynthesis. To identify further candidates for the activation of the *sylA* gene, a more complete knowledge of the SalA regulon would be helpful. This could be achieved by transcriptome analysis using the newly available commercial *Pss* B728a whole genome gene chip (Roche NimbleGen Inc.).

The regulation of syringolin A biosynthesis is complex and known to be influenced by factors mimicking *in planta* conditions. In chapter 3 we presented data indicating that oxygen concentration is involved in syringolin A biosynthesis, i.e. that microaerobic conditions are activating syringolin A biosynthesis. We demonstrated further, that this effect is not mediated by the Anr orthologue of *Pss* B301D-R. Adaptation of the metabolism to different oxygen levels is an essential feature for bacteria to survive in fluctuating environments. In *P. aeruginosa* for example, the Fnr-type oxygen sensor Anr (anaerobic regulator of arginine deaminase and nitrate reductase) is known to mediate the regulation of many pathways activated or repressed by low oxygen tension (Trunk et al., 2010). Oxygen limitation was also identified as an inducer for the production of secondary metabolites by microbes in culture (Clark et al., 1995; Rollins et al., 1988). The biological relevance of oxygen sensitivity in phytopathogens as *Pss* is not obvious because *in planta*, on the leaf surface or in the apoplast, oxygen is not expected to be limited. On the contrary, the atmosphere in the intercellular space may exhibit high oxygen concentrations during photosynthesis, although, little is known about the absolute oxygen concentrations inside leaves. To identify additional genes involved in syringolin A biosynthesis, a mutant screen would be helpful. However, this approach is hampered by the absence of an efficient screening method for syringolin A-negative mutants.

Eventually, we have to consider that mechanisms triggered *in vitro* by oxygen limitation may be regulated differently *in planta*. Because the phyllosphere is a very ephemeral habitat

with continuously fluctuating physical conditions, it may be very challenging to elucidate all the regulation mechanisms *in vitro*.

In chapter 4 we described how syringolin A counteracts stomatal immunity and antagonizes the SA-dependent defense pathway by inhibition of the host proteasome. It is known that certain virulence factors produced by certain pathovars of *P. syringae* rely on the action of the host proteasome for their mode of action. An example is *P. syringae* pv. *tomato* (*Pto*) DC3000, which secretes the small-molecule virulence factor coronatine and the type III effector HopAB2 (formerly named AvrPtoB). Coronatine is a molecular mimic of the plant defense hormone isoleucine-conjugated jasmonic acid (Ile-JA) (Bender et al., 1999). The JA-dependent defense pathway is directed against necrotrophic pathogens and in Arabodopsis is antagonistic to the SA-dependent defense pathway which is directed against biotrophic or hemibiotrophic pathogens like *P. syringae* (Brooks et al., 2005). Thus, coronatine will suppress the SA pathway in a as yet unknown manner and therefore be beneficial to its producer. In Arabidopsis, the JA signalling pathway involves *CORONATINE-INSENSITIVE-1* (*COI1*), which encodes an F-box protein that is part of the SCF^{COI1} E3 ubiquitin ligase which targets repressors of JA-dependent gene activation to proteasomal destruction upon JA signalling (Chini et al., 2007; Thines et al., 2007).

The type III effector HopAB2 is an E3 ubiquitin ligase encoded in the genome of *Pto* which targets the tomato protein kinase Fen, which has a role in pathogen defense, for proteasomal destruction, and thus leads to disease susceptibility of the host plant (Rosebrock et al., 2007). The concept that a pathogen would rely on a functional host proteasome to thwart defense reactions in the host plant, while concurrently producing a compound as syringolin A which acts as a proteasome inhibitor is somewhat contradicting. Interestingly, in the fully sequenced genome of *Pss* B728a, neither coronatine synthetase genes nor a HopAB2 encoding gene is present (Sarkar et al., 2006), perhaps indicating that molecules with these distinct modes of action exclude each other within the same bacterial strain. If not, it would be reasonable that the two types of molecules are exclusively expressed depending on time and conditions. Comparative analysis of the genomes of *Pss*

B728a and *Pst* DC3000 revealed many genes unique to one or the other pathovar. These differences seem to reflect distinct behavioral traits of the organisms, such as e.g. unique genes in *Pss* contributing to epiphytic fitness (Feil et al., 2005). Whether strains producing syringolin A have different life strategies from the ones producing factors that subvert the host proteasome for their own benefit is at present not clear. Mutational analyses and more comprehensive comparative genome analyses of strains with distinct host plants as well as of strains with the same hosts but different life strategies could reveal more information about genes related to specific host-pathogen interaction.

6.

LITERATURE

Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics Int.* **11**, 36-42.

Acharya, B. R. and Assmann, S. M. (2009). Hormone interactions in stomatal function. *Plant Mol. Biol.* **69**, 451-462.

Amrein, H., Makart, S., Granado, J., Shakya, R., Schneider-Pokorny, J. and Dudler, R. (2004). Functional analysis of genes involved in the synthesis of syringolin A by *Pseudomonas syringae* pv. *syringae* B301D-R. *Mol. Plant-Microbe Interact.* **17**, 90-97.

Ashorpour, M., Kazempour, M. N. and Ramezanie, M. (2008). Bacterial canker of olives caused by of *Pseudomonas syringae* pv. *syringae* in Iran. *Sci. Hortic.* **118**, 128-131.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. and Struhl, K. (1987). Current protocols in molecular biology. New York: Wiley and Sons.

Backman, P. A. and Devay, J. E. (1971). Studies on mode of action and biogenesis of phytotoxin syringomycin. *Physiol. Plant Pathol.* **1**, 215-224.

Ballio, A., Mauri, M., Chain, E. B., Deleo, P., Tonolo, A. and Erlanger, B. F. (1964). Fusicoccin - new wilting toxin produced by *Fusicoccum amygdali* Del. *Nature* **203**, 297.

Bauer, C. E., Elsen, S. and Bird, T. H. (1999). Mechanisms for redox control of gene expression. *Annu. Rev. Microbiol.* **53**, 495-523.

Baunsgaard, L., Fuglsang, A. T., Jahn, T., Korthout, H., de Boer, A. H. and Palmgren, M. G. (1998). The 14-3-3 proteins associate with the plant plasma membrane H(+)-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. *Plant J.* **13**, 661-671.

Bender, C. L., Alarcon-Chaidez, F. and Gross, D. C. (1999). *Pseudomonas syringae* phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* **63**, 266-292.

Blumer, C. and Haas, D. (2000). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch. Microbiol.* **173**, 170-177.

Blumer, C., Heeb, S., Pessi, G. and Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14073-14078.

Boller, T. and Felix, G. (2009). A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* **60**, 379-406.

Bradbury, J. F. (1986). *Pseudomonas syringae* pv. *syringae*. In *Guide to plant pathogenic bacteria*, (ed. C. I. M. Institute), pp. 175-177. Kew, England.

Brooks, D. M., Bender, C. L. and Kunkel, B. N. (2005). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Mol. Plant Pathol.* **6**, 629-639.

Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987). XL1-Blue - a high-efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *BioTechniques* **5**, 376-379.

Cao, H., Bowling, S. A., Gordon, A. S. and Dong, X. N. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired-resistance. *Plant Cell* **6**, 1583-1592.

Cazorla, F. M., Tores, J. A., Olalla, L., Perez-Garcia, A., Farre, J. M. and de Vicente, A. (1998). Bacterial apical necrosis of mango in southern Spain: A disease caused by *Pseudomonas syringae* pv. *syringae*. *Phytopathology* **88**, 614-620.

Chatterjee, A., Cui, Y. Y., Yang, H. L., Collmer, A., Alfano, J. R. and Chatterjee, A. K. (2003). GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol. Plant-Microbe Interact.* **16**, 1106-1117.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J. M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F. M., Ponce, M. R. et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**, 666-671.

Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B. J. (2006). Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* **124**, 803-814.

Christensen, B. B., Sternberg, C., Andersen, J. B., Palmer, R. J., Nielsen, A. T., Givskov, M. and Molin, S. (1999). Molecular tools for the study of biofilm physiology. In *Methods Enzymol.*, pp. 20-42. San Diego: Academic Press.

Christiansen, G., Philmus, B., Hemscheidt, T. and Kurmayer, R. (2011). Genetic variation of adenylation domains of the anabaenopeptin synthesis operon and evolution of substrate promiscuity. *J. Bacteriol.* **193**, 3822-3831.

Churchill, M. E. A. and Chen, L. (2011). Structural basis of acyl-homoserine lactone-dependent signaling. *Chem. Rev.* **111**, 68-85.

Clark, G. J., Langley, D. and Bushell, M. E. (1995). Oxygen limitation can induce microbial secondary metabolite formation - investigations with miniature electrodes in shaker and bioreactor culture. *Microbiology-Uk* **141**, 663-669.

Clerc, J., Groll, M., Illich, D. J., Bachmann, A. S., Huber, R., Schellenberg, B., Dudler, R. and Kaiser, M. (2009). Synthetic and structural studies on syringolin A and B reveal critical determinants of selectivity and potency of proteasome inhibition. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 6507-6512.

Coleman, C. S., Rocetes, J. P., Park, D. J., Wallick, C. J., Warn-Cramer, B. J., Michel, K., Dudler, R. and Bachmann, A. S. (2006). Syringolin A, a new plant elicitor from the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*, inhibits the proliferation of neuroblastoma and ovarian cancer cells and induces apoptosis. *Cell Prolif.* **39**, 599-609.

Cui, H., Xiang, T. and Zhou, J.-M. (2009). Plant immunity: a lesson from pathogenic bacterial effector proteins. *Cell. Microbiol.* **11**, 1453-1461.

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. et al. (1994). A central role of salicylic acid in plant disease resistance. *Science* **266**, 1247-1250.

Dillon, S. C. and Dorman, C. J. (2010). Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. *Nat. Rev. Microbiol.* **8**, 185-195.

Dong, W. B., Nowara, D. and Schweizer, P. (2006). Protein polyubiquitination plays a role in basal host resistance of barley. *Plant Cell* **18**, 3321-3331.

Dong, X. N. (2001). Genetic dissection of systemic acquired resistance. *Curr. Opin. Plant Biol.* **4**, 309-314.

Dulla, G. F. J. and Lindow, S. E. (2009). Acyl-homoserine lactone-mediated cross talk among epiphytic bacteria modulates behavior of *Pseudomonas syringae* on leaves. *Isme J.* **3**, 825-834.

Dumenyo, C. K., Mukherjee, A., Chun, W. and Chatterjee, A. K. (1998). Genetic and physiological evidence for the production of N-acyl homoserine lactones by *Pseudomonas syringae* pv. *syringae* and other fluorescent plant pathogenic *Pseudomonas* species. *Eur. J. Plant Pathol.* **104**, 569-582.

Dutta, R., Qin, L. and Inouye, M. (1999). Histidine kinases: diversity of domain organization. *Mol. Microbiol.* **34**, 633-640.

Engebrecht, J. and Silverman, M. (1984). Identification of genes and gene-products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4154-4158.

Feil, H., Feil, W. S., Chain, P., Larimer, F., DiBartolo, G., Copeland, A., Lykidis, A., Trong, S., Nolan, M., Goltsman, E. et al. (2005). Comparison of the complete genome sequences of *Pseudomonas syringae* pv. *syringae* B728a and pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 11064-11069.

Finan, T. M., Kunkel, B., Devos, G. F. and Signer, E. R. (1986). 2nd symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamin synthesis genes. *J. Bacteriol.* **167**, 66-72.

Finking, R. and Marahiel, M. A. (2004). Biosynthesis of nonribosomal peptides. *Annu. Rev. Microbiol.* **58**, 453-488.

Fischbach, M. A. and Walsh, C. T. (2006). Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: Logic, machinery, and mechanisms. *Chem. Rev.* **106**, 3468-3496.

Fleming, J. A., Lightcap, E. S., Sadis, S., Thoroddsen, V., Bulawa, C. E. and Blackman, R. K. (2002). Complementary whole-genome technologies reveal the cellular response to proteasome inhibition by PS-341. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 1461-1466.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **9**, 275-296.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S. Y., Cutler, S. R., Sheen, J., Rodriguez, P. L. and Zhu, J. K. (2009). In vitro reconstitution of an abscisic acid signalling pathway. *Nature* **462**, 660-664.

Gesner-Apter, S. and Carmeli, S. (2008). Three novel metabolites from a bloom of the cyanobacterium *Microcystis* sp. *Tetrahedron* **64**, 6628-6634.

Golemi, D., Maveyraud, L., Vakulenko, S., Samama, J. P. and Mobashery, S. (2001). Critical involvement of a carbamylated lysine in catalytic function of class D beta-lactamases. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 14280-14285.

Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y. and Collmer, A. (1996). Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8**, 1095-1105.

Goritschnig, S., Zhang, Y. L. and Li, X. (2007). The ubiquitin pathway is required for innate immunity in Arabidopsis. *Plant J.* **49**, 540-551.

Grant, S. G. N., Jessee, J., Bloom, F. R. and Hanahan, D. (1990). Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4645-4649.

Green, J., Crack, J. C., Thomson, A. J. and LeBrun, N. E. (2009). Bacterial sensors of oxygen. *Curr. Opin. Microbiol.* **12**, 145-151.

Groll, M., Schellenberg, B., Bachmann, A. S., Archer, C. R., Huber, R., Powell, T. K., Lindow, S., Kaiser, M. and Dudler, R. (2008). A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. *Nature* **452**, 755-758.

Gross, D. C. (1985). Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. *J. Appl. Bacteriol.* **58**, 167-174.

Gross, D. C. (1991). Molecular and genetic-analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annu. Rev. Phytopathol.* **29**, 247-278.

Gross, D. C. and DeVay, J. E. (1977). Role of syringomycin in holcus spot of maize and systemic necrosis of cowpea caused by *Pseudomonas syringae*. *Physiol. Plant Pathol.* **11**, 1-11.

Gruenewald, S., Mootz, H. D., Stehmeier, P. and Stachelhaus, T. (2004). *In vivo* production of artificial nonribosomal peptide products in the heterologous host *Escherichia coli*. *Appl. Environ. Microbiol.* **70**, 3282-3291.

Gudesblat, G. E., Torres, P. S. and Vojnov, A. A. (2009). *Xanthomonas campestris* overcomes arabidopsis stomatal innate immunity through a DSF cell-to-cell signal-regulated virulence factor. *Plant Physiol.* **149**, 1017-1027.

Guenzi, E., Galli, G., Grgurina, I., Gross, D. C. and Grandi, G. (1998). Characterization of the syringomycin synthetase gene cluster - A link between prokaryotic and eukaryotic peptide synthetases. *J. Biol. Chem.* **273**, 32857-32863.

Guo, M., Tian, F., Wamboldt, Y. and Alfano, J. R. (2009). The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant Immunity. *Mol. Plant-Microbe Interact.* **22**, 1069-1080.

Harada, K., Fujii, K., Shimada, T., Suzuki, M., Sano, H., Adachi, K. and Carmichael, W. W. (1995). Two cyclic peptides, anabaenopeptins, a third group of bioactive compounds from the cyanobacterium *Anabaena flos-aquae* NRC-525-17. *Tetrahedron Lett.* **36**, 1511-1514.

Hassa, P., Granado, J., Freydl, E., Waspi, U. and Dudler, R. (2000). Syringolin-mediated activation of the Pir7b esterase gene in rice cells is suppressed by phosphatase inhibitors. *Mol. Plant-Microbe Interact.* **13**, 342-346.

He, P., Shan, L. and Sheen, J. (2007). Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. *Cell. Microbiol.* **9**, 1385-1396.

Heeb, S. and Haas, D. (2001). Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Mol. Plant-Microbe Interact.* **14**, 1351-1363.

Hirano, S. S., Ostertag, E. M., Savage, S. A., Baker, L. S., Willis, D. K. and Upper, C. D. (1997). Contribution of the regulatory gene *lemA* to field fitness of *Pseudomonas syringae* pv. *syringae*. *Appl. Environ. Microbiol.* **63**, 4304-4312.

Hirano, S. S. and Upper, C. D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev.* **64**, 624-653.

Hopwood, D. A. (1997). Genetic contributions to understanding polyketide synthases. *Chem. Rev.* **97**, 2465-2497.

Hrabak, E. M. and Willis, D. K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J. Bacteriol.* **174**, 3011-3020.

Hrabak, E. M. and Willis, D. K. (1993). Involvement of the *lemA* gene in production of syringomycin and protease by *Pseudomonas syringae* pv. *syringae*. *Mol. Plant-Microbe Interact.* **6**, 368-375.

Hull, G., Garrido, J. M. G., Parcy, F., Menossi, M., MartinezIzquierdo, J. A. and Gallois, P. (1996). Use of the *lacZ* reporter gene as an internal control for GUS activity in microprojectile bombarded plant tissue. *Plant Science* **120**, 153-160.

Huo, Y. X., Zhang, Y. T., Xiao, Y., Zhang, X. D., Buck, M., Kolb, A. and Wang, Y. P. (2009). IHF-binding sites inhibit DNA loop formation and transcription initiation. *Nucleic Acids Res.* **37**, 3878-3886.

Imker, H. J., Walsh, C. T. and Wuest, W. M. (2009). SylC catalyzes ureido-bond formation during biosynthesis of the proteasome inhibitor syringolin A. *J. Am. Chem. Soc.* **131**, 18263-18265.

Jones, J. D. G. and Dangl, J. L. (2006). The plant immune system. *Nature* **444**, 323-329.

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T. et al. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* **36**, D480-D484.

Kinscherf, T. G. and Willis, D. K. (1999). Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone biosynthetic gene *ahlI*. *J. Bacteriol.* **181**, 4133-4136.

Kitten, T., Kinscherf, T. G., McEvoy, J. L. and Willis, D. K. (1998). A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. *Mol. Microbiol.* **28**, 917-929.

Kleerebezem, M., Quadri, L. E. N., Kuipers, O. P. and deVos, W. M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol. Microbiol.* **24**, 895-904.

Klusener, B., Young, J. J., Murata, Y., Allen, G. J., Mori, I. C., Hugouvieux, V. and Schroeder, J. I. (2002). Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. *Plant Physiol.* **130**, 2152-2163.

Koebnik, R., Kruger, A., Thieme, F., Urban, A. and Bonas, U. (2006). Specific binding of the Xanthomonas campestris pv. vesicatoria AraC-type transcriptional activator HrpX to plant-inducible promoter boxes. *J. Bacteriol.* **188**, 7652-7660.

Koornneef, M., Reuling, G. and Karssen, C. M. (1984). The isolation and characterization of abscisic-acid insensitive mutants of *Arabidopsis thaliana*. *Physiol. Plant.* **61**, 377-383.

Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C. and Walsh, C. T. (1996). A new enzyme superfamily - The phosphopantetheinyl transferases. *Chemistry & Biology* **3**, 923-936.

Lapouge, K., Schubert, M., Allain, F. H. T. and Haas, D. (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol. Microbiol.* **67**, 241-253.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. and Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic-synthesis and suppression of black root-rot of tobacco. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1562-1566.

Lee, S., Choi, H., Suh, S., Doo, I. S., Oh, K. Y., Choi, E. J., Taylor, A. T. S., Low, P. S. and Lee, Y. (1999). Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol.* **121**, 147-152.

Little, E. L., Bostock, R. M. and Kirkpatrick, B. C. (1998). Genetic characterization of *Pseudomonas syringae* pv. *syringae* strains from stone fruits in California. *Appl. Environ. Microbiol.* **64**, 3818-3823.

Liu, J., Elmore, J. M., Fuglsang, A. T., Palmgren, M. G., Staskawicz, B. J. and Coaker, G. (2009). RIN4 functions with plasma membrane H(+)-ATPases to regulate stomatal apertures during pathogen attack. *PLoS Biol.* **7**.

Lu, S. E., Scholz-Schroeder, B. K. and Gross, D. C. (2002a). Characterization of the *salA*, *syrF*, and *syrG* regulatory genes located at the right border of the syringomycin gene cluster of *Pseudomonas syringae pv. syringae*. *Mol. Plant-Microbe Interact.* **15**, 43-53.

Lu, S. E., Scholz-Schroeder, B. K. and Gross, D. C. (2002b). Construction of pMEKm12, an expression vector for protein production in *Pseudomonas syringae*. *FEMS Microbiol. Lett.* **210**, 115-121.

Lu, S. E., Wang, N., Wang, J. L., Chen, Z. J. and Gross, D. C. (2005). Oligonucleotide microarray analysis of the salA regulon controlling phytotoxin production by *Pseudomonas syringae pv. syringae*. *Mol. Plant-Microbe Interact.* **18**, 324-333.

Macchi, R., Montesissa, L., Murakami, K., Ishihama, A., de Lorenzo, V. and Bertoni, G. (2003). Recruitment of sigma54-RNA polymerase to the Pu promoter of *Pseudomonas putida* through integration host factor-mediated positioning switch of alpha subunit carboxyl-terminal domain on an UP-like element. *J. Biol. Chem.* **278**, 27695-27702.

Mansfield, J. W. (2009). From bacterial avirulence genes to effector functions via the hrp delivery system: an overview of 25 years of progress in our understanding of plant innate immunity. *Mol. Plant Pathol.* **10**, 721-734.

Marahiel, M. A., Stachelhaus, T. and Mootz, H. D. (1997). Modular peptide synthetases involved in nonribosomal peptide synthesis. *Chem. Rev.* **97**, 2651-2673.

Marre, E. (1979). Fusicoccin - tool in plant physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **30**, 273-288.

Martin-Sanz, A., Luis Palomo, J., Perez de la Vega, M. and Caminero, C. (2011). Identification of pathovars and races of *Pseudomonas syringae*, the main causal agent of bacterial disease in pea in North-Central Spain, and the search for disease resistance. *Eur. J. Plant Pathol.* **129**, 57-69.

Matthew, S., Ross, C., Paul, V. J. and Luesch, H. (2008). Pompanopeptins A and B, new cyclic peptides from the marine cyanobacterium *Lyngbya confervoides*. *Tetrahedron* **64**, 4081-4089.

Maveyraud, L., Golemi, D., Kotra, L. P., Tranier, S., Vakulenko, S., Mobashery, S. and Samama, J. P. (2000). Insights into class D beta-lactamases are revealed by the crystal structure of the OXA10 enzyme from *Pseudomonas aeruginosa*. *Structure* **8**, 1289-1298.

Meier, J. L. and Burkart, M. D. (2009). Synthetic probes for polyketide and nonribosomal peptide biosynthetic enzymes. In *Complex Enzymes in Microbial Natural Product Biosynthesis, Part A: Overview Articles and Peptides*, vol. 458 (ed. D. A. Hopwood), pp. 219-254. San Diego: Elsevier Academic Press Inc.

Meiners, S., Heyken, D., Weller, A., Ludwig, A., Stangl, K., Kloetzel, P. M. and Kruger, E. (2003). Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of mammalian proteasomes. *J. Biol. Chem.* **278**, 21517-21525.

Melcher, K., Ng, L. M., Zhou, X. E., Soon, F. F., Xu, Y., Suino-Powell, K. M., Park, S. Y., Weiner, J. J., Fujii, H., Chinnusamy, V. et al. (2009). A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. *Nature* **462**, 602-608.

Melotto, M., Underwood, W. and He, S. Y. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu. Rev. Phytopathol.* **46**, 101-122.

Melotto, M., Underwood, W., Koczan, J., Nomura, K. and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* **126**, 969-980.

Melville, S. B. and Gunsalus, R. P. (1996). Isolation of an oxygen-sensitive FNR protein of *Escherichia coli*: interaction at activator and repressor sites of FNR-controlled genes. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 1226-1231.

Metraux, J.-P., Jackson, R. W., Schnettler, E. and Goldbach, R. W. (2009). Plant pathogens as suppressors of host defense. In *Plant Innate Immunity*, vol. 51 (ed. L. C. VanLoon), pp. 39-89.

Métraux, J. P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J. and Ward, E. (1991). Induced systemic resistance in cucumber in response to 2,6-dichloro-isonicotinic acid and pathogens. In *Advances in Molecular Genetics of Plant-Microbe Interactions.*, vol. 1 (ed. H. Hennecke and D. P. S. Verma), pp. 432-439. Dordrecht: Kluwer.

Michel, K., Abderhalden, O., Bruggmann, R. and Dudler, R. (2006). Transcriptional changes in powdery mildew infected wheat and Arabidopsis leaves undergoing syringolin-triggered hypersensitive cell death at infection sites. *Plant Mol. Biol.* **62**, 561-578.

Miller, J. H. (1972). Experiments in Molecular Genetics. *Cold Spring Harbor Laboratory* **Cold Spring Harbor, New York**.

Miller, M. B. and Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**, 165-199.

Miyazono, K., Miyakawa, T., Sawano, Y., Kubota, K., Kang, H. J., Asano, A., Miyauchi, Y., Takahashi, M., Zhi, Y. H., Fujita, Y. et al. (2009). Structural basis of abscisic acid signalling. *Nature* **462**, 609-614.

Mo, Y.-Y. and Gross, D. C. (1991b). Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.* **173**, 5784-5792.

Mo, Y. Y. and Gross, D. C. (1991a). Expression *in vitro* and during plant pathogenesis of the *syrB* gene required for syringomycin production by *Pseudomonas syringae* pathovar *syringae*. *Mol. Plant-Microbe Interact.* **4**, 28-36.

Moes, D., Himmelbach, A., Korte, A., Haberer, G. and Grill, E. (2008). Nuclear localization of the mutant protein phosphatase abi1 is required for insensitivity towards ABA responses in Arabidopsis. *Plant J.* **54**, 806-819.

Muller, D., Krick, A., Kehraus, S., Mehner, C., Hart, M., Kupper, F. C., Saxena, K., Prinz, H., Schwalbe, H., Janning, P. et al. (2006). Brunsvicamides A-C: Sponge-related cyanobacterial peptides with *Mycobacterium tuberculosis* protein tyrosine phosphatase inhibitory activity. *J. Med. Chem.* **49**, 4871-4878.

Munch, R., Hiller, K., Grote, A., Scheer, M., Klein, J., Schobert, M. and Jahn, D. (2005). Virtual footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* **21**, 4187-4189.

Murakami, M., Shin, H. J., Matsuda, H., Ishida, K. and Yamaguchi, K. (1997). A cyclic peptide, anabaenopeptin B, from the cyanobacterium *Oscillatoria agardhii*. *Phytochemistry* **44**, 449-452.

Nishimura, N., Hitomi, K., Arvai, A. S., Rambo, R. P., Hitomi, C., Cutler, S. R., Schroeder, J. I. and Getzoff, E. D. (2009). Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. *Science* **326**, 1373-1379.

Oka, M., Nishiyama, Y., Ohta, S., Kamei, H., Konishi, M., Miyaki, T., Oki, T. and Kawaguchi, H. (1988a). Glidobactins A, B and C, new antitumor antibiotics. I. Production, isolation, chemical properties and biological activity. *J. Antibiot.* **41**, 1331-1337.

Oka, M., Yaginuma, K., Numata, K., Konishi, M., Oki, T. and Kawaguchi, H. (1988b). Glidobactin-A, glidobactin-B and glidobactin-C, new antitumor antibiotics .II. Structure elucidation. *J. Antibiot.* **41**, 1338-1350.

Okumura, H. S., Philmus, B., Portmann, C. and Hemscheidt, T. K. (2009). Homotyrosine-containing cyanopeptolins 880 and 960 and anabaenopeptins 908 and 915 from *Planktothrix agardhii* CYA 126/8. *J. Nat. Prod.* **72**, 172-176.

Olivari, C., Meanti, C., De Michelis, M. I. and Rasi-Caldogno, F. (1998). Fusicoccin binding to its plasma membrane receptor and the activation of the plasma membrane H(+)-ATPase - IV. Fusicoccin induces the association between the plasma membrane H(+)-ATPase and the fusicoccin receptor. *Plant Physiol.* **116**, 529-537.

Pabo, C. O. and Sauer, R. T. (1992). Transcription factors - structural families and principles of DNA recognition. *Annu. Rev. Biochem.* **61**, 1053-1095.

Perraud, A. L., Rippe, K., Bantscheff, M., Glocker, M., Lucassen, M., Jung, K., Sebald, W., Weiss, V. and Gross, R. (2000). Dimerization of signalling modules of the EvgAS and BvgAS phosphorelay systems. *Biochim. Biophys. Acta* **1478**, 341-354.

Pieterse, C. M. J. and Dicke, M. (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends Plant Sci.* **12**, 564-569.

Quandt, J. and Hynes, M. F. (1993). Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* **127**, 15-21.

Quinones, B., Dulla, G. and Lindow, S. E. (2005). Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* **18**, 682-693.

Quinones, B., Pujol, C. J. and Lindow, S. E. (2004). Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* **17**, 521-531.

Rahimian, H. (1995). The occurrence of bacterial red streak of sugarcane caused by *Pseudomonas syringae* pv. *syringae* in iran. *J. Phytopathol.* **143**, 321-324.

Ramel, C., Tobler, M., Meyer, M., Bigler, L., Ebert, M. O., Schellenberg, B. and Dudler, R. (2009). Biosynthesis of the proteasome inhibitor syringolin A: the ureido group joining two amino acids originates from bicarbonate. *BMC Biochem.* **10**.

Reimmann, C., Hofmann, C., Mauch, F. and Dudler, R. (1995). Characterization of a rice gene induced by *Pseudomonas syringae* pv. *syringae*: Requirement for the bacterial *lemA* gene function. *Physiol. Mol. Plant Pathol.* **46**, 71-81.

Rich, J. J., Hirano, S. S. and Willis, D. K. (1992). Pathovar-specific requirement for the *Pseudomonas syringae lemA* gene in disease lesion formation. *Appl. Environ. Microbiol.* **58**, 1440-1446.

Rich, J. J., Kinscherf, T. G., Kitten, T. and Willis, D. K. (1994). Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J. Bacteriol.* **176**, 7468-75.

Rollins, M. J., Jensen, S. E. and Westlake, D. W. S. (1988). Effect of aeration on antibiotic production by *Streptomyces clavuligerus*. *J. Ind. Microbiol. Biotechnol.* **3**, 357-364.

Rosebrock, T. R., Zeng, L. R., Brady, J. J., Abramovitch, R. B., Xiao, F. M. and Martin, G. B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. *Nature* **448**, 370-374.

Salmond, G. P. C. (1994). Secretion of extracellular virulence factors by plant-pathogenic bacteria. *Annu. Rev. Phytopathol.* **32**, 181-200.

Santiago, J., Dupeux, F., Round, A., Antoni, R., Park, S. Y., Jamin, M., Cutler, S. R., Rodriguez, P. L. and Marquez, J. A. (2009a). The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature* **462**, 665-668.

Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F., Park, S. Y., Marquez, J. A., Cutler, S. R. and Rodriguez, P. L. (2009b). Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. *Plant J.* **60**, 575-588.

Sarkar, S. F., Gordon, J. S., Martin, G. B. and Guttman, D. S. (2006). Comparative genomics of host-specific virulence in *Pseudomonas syringae*. *Genetics* **174**, 1041-1056.

Sawers, G. (1999). The aerobic/anaerobic interface. *Curr. Opin. Microbiol.* **2**, 181-187.

Schellenberg, B., Bigler, L. and Dudler, R. (2007). Identification of genes involved in the biosynthesis of the cytotoxic compound glidobactin from a soil bacterium. *Environ. Microbiol.* **9**, 1640-1650.

Schellenberg, B., Ramel, C. and Dudler, R. (2010). *Pseudomonas syringae* virulence factor syringolin A counteracts stomatal immunity by proteasome inhibition. *Mol. Plant-Microbe Interact.* **23**, 1287-1293.

Schmidt, E. W., Harper, M. K. and Faulkner, D. J. (1997). Mozamides A and B, cyclic peptides from a theonellid sponge from Mozambique. *J. Nat. Prod.* **60**, 779-782.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D. et al. (2000). Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J. Bacteriol.* **182**, 1215-1225.

Segonzac, C. and Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria. *Curr. Opin. Microbiol.* **14**, 54-61.

Senior, E., Bull, A. T. and Slater, J. H. (1976). Enzyme evolution in a microbial community growing on herbicide Dalapon. *Nature* **263**, 476-479.

Simon, R., Priefer, U. and Puhler, A. (1983). A broad host range mobilization system for *in vivo* geneic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio-Technology* **1**, 784-791.

Smith, J. A. and Métraux, J. P. (1991). *Pseudomonas syringae* pathovar *syringae* induces systemic resistance to *Pyricularia oryzae* in rice. *Physiol. Mol. Plant Pathol.* **39**, 451-461.

Spoel, S. H., Mou, Z. L., Tada, Y., Spivey, N. W., Genschik, P. and Dong, X. N. A. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* **137**, 860-872.

Stachelhaus, T. and Marahiel, M. A. (1995). Modular structure of genes encoding multifunctional peptide synthetases required for nonribosomal peptide-synthesis. *FEMS Microbiol. Lett.* **125**, 3-14.

Stachelhaus, T., Mootz, H. D., Bergendahl, V. and Marahiel, M. A. (1998). Peptide bond formation in nonribosomal peptide biosynthesis - Catalytic role of the condensation domain. *J. Biol. Chem.* **273**, 22773-22781.

Staskawicz, B., Dahlbeck, D., Keen, N. and Napoli, C. (1987). Molecular characterization of cloned avirulence genes from race-0 and race-1 of *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* **169**, 5789-5794.

Teeri, T. H., Lehvaslaiho, H., Franck, M., Uotila, J., Heino, P., Palva, E. T., Vanmontagu, M. and Herreraestrella, L. (1989). Gene Fusions to *LacZ* reveal new expression patterns of chimeric genes in transgenic plants. *EMBO J.* **8**, 343-350.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G. H., Nomura, K., He, S. Y., Howe, G. A. and Browse, J. (2007). JAZ repressor proteins are targets of the SCFCO11 complex during jasmonate signalling. *Nature* **448**, 661-665.

Trunk, K., Benkert, B., Quack, N., Munch, R., Scheer, M., Garbe, J., Jansch, L., Trost, M., Wehland, J., Buer, J. et al. (2010). Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. *Environ. Microbiol.* **12**, 1719-1733.

Vierstra, R. D. (2009). The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat. Rev. Mol. Cell Biol.* **10**, 385-397.

Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., Leung, J., Rodriguez, P. L., Lauriere, C. and Merlot, S. (2009). Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in arabidopsis. *Plant Cell* **21**, 3170-3184.

Voisard, C., Bull, C. T., Keel, C., Laville, J., Maurhofer, M., Schnider, U., Défago, G. and Haas, D. (1994). Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In *Molecular ecology of rhizosphere microorganisms.*, (ed. O. G. F. D. D. and B. B), pp. 67-89. Weinheim, Germany: VCH Publishers.

von Döhren, H., Dieckmann, R. and Pavela-Vrancic, M. (1999). The nonribosomal code. Chem. and Biol. **6**, R273-R279.

Walther, T., Renner, S., Waldmann, H. and Arndt, H.-D. (2009). Synthesis and Structure-Activity Correlation of a Brunsvicamide-Inspired Cyclopeptide Collection. *ChemBioChem* **10**, 1153-1162.

Wang, N., Lu, S. E., Records, A. R. and Gross, D. C. (2006). Characterization of the transcriptional activators SalA and SyrF, which are required for syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.* **188**, 3290-3298.

Wäspi, U., Blanc, D., Winkler, T., Ruedi, P. and Dudler, R. (1998a). Syringolin, a novel peptide elicitor from *Pseudomonas syringae* pv. *syringae* that induces resistance to *Pyricularia oryzae* in rice. *Mol. Plant-Microbe Interact.* **11**, 727-733.

Wäspi, U., Hassa, P., Staempfli, A., Molleyres, L.-P., Winkler, T. and Dudler, R. (1999). Identification and structure of a family of syringolin variants: Unusual cyclic peptides from *Pseudomonas syringae* pv. *syringae* that elicit defense responses in rice. *Microbiol. Res.* **154**, 1-5.

Wäspi, U., Misteli, B., Hasslacher, M., Jandrositz, A., Kohlwein, S. D., Schwab, H. and Dudler, R. (1998b). The defense-related rice gene *Pir7b* encodes an "alpha/beta hydrolase fold" protein exhibiting esterase activity towards naphthol AS-esters. *Eur. J. Biochem.* **254**, 32-37.

Wäspi, U., Schweizer, P. and Dudler, R. (2001). Syringolin reprograms wheat to undergo hypersensitive cell death in a compatible interaction with powdery mildew. *Plant Cell* **13**, 153-161.

Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L. and Salmond, G. P. C. (2001). Quorum-sensing in gram-negative bacteria. *FEMS Microbiol. Rev.* **25**, 365-404.

Williams, D. E., Craig, M., Holmes, C. F. B. and Andersen, R. J. (1996). Ferintoic acids A and B, new cyclic hexapeptides from the freshwater cyanobacterium *Microcystis aeruginosa*. *J. Nat. Prod.* **59**, 570-575.

Willis, D. K., Hrabak, E. M., Rich, J. J., Barta, T. M., Lindow, S. E. and Panopoulos, N. J. (1990). Isolation and characterization of a *Pseudomonas syringae* pathovar *syringae* mutant deficient in lesion formation on bean. *Mol. Plant-Microbe Interact.* **3**, 149-156.

Xu, G. W. and Gross, D. C. (1988). Physical and functional analyses of the *syrA* and *syrB* genes involved in syringomycin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.* **170**, 5680-8.

Young, J. M. (1991). Pathogenicity and identification of the lilac pathogen, *Pseudomonas syringae* pv. *syringae* van Hall 1902. *Ann. Appl. Biol.* **118**, 283-298.

Zhang, W., He, S. Y. and Assmann, S. M. (2008). The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. *Plant J.* **56**, 984-996.

Acknowledgements

THANK YOU…

- Prof. Dr. Leo Eberl, for being referee and chair of my thesis-committee.
  I enjoyed the work in a field that is an interface of microbiology and plant biology.

- PD Dr. Cornelia Reimmann, for kindly agreeing to be co-referee and part of my thesis-committee and for especially arriving from Lausanne for my exam.

- Prof. Dr. Robert Dudler, for giving me the opportunity to do my PhD in your group on this fascinating and versatile project, to broaden my horizon, learn a plethora of things and grow upon the challenge. Thank you for being an excellent and supportive supervisor, for your patience and the ability to motivate in the difficult stages of the project. You were always an example in your innovative way of thinking and your enthusiasm for all the fascinating questions of science.

- Zsuzsa Hasenkamp, for being our helpful, reliable source of Syringolin, competent cells and other imperatives, without that a great deal of our science would not be possible. I enjoyed all our nice chats and discussions, which were always very interesting, despite or just because we are quite different. Thank you for your hearty, cheerful and sympathetic nature!

- Barbara Schellenberg, for teaching me more or less all the basics, I will never forget how you showed me to pour my first gel as a master student. For your interest, patience and time for all the important end less important problems. Thank you for being an excellent lab-mate and just a great person.

- members of the Dudler group, it was great to encounter -although in this rather small group- so many good colleagues: Zsuzsa, Barbara, Knubi, Michel, Micha, Martin, Nando, Julia, David, Leonie, Alexey, Shaohui, Mirjam and Silvia. Thank you for fruitful teamwork and all the good moments! It was nice to work with you together and share the every day lab-life.

- bunch of great Keller group people, I somewhat regret that I didn't found more time to spend with you!

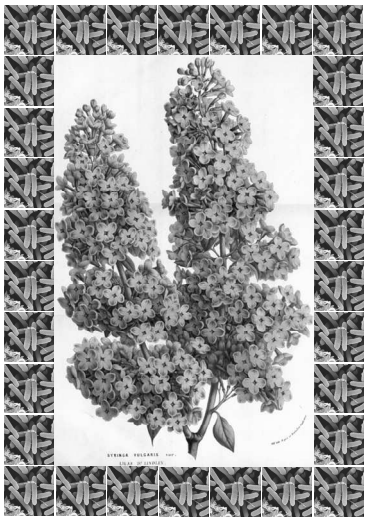
THANK YOU…

- Caroline with Noemie, Rahel & Jari, for your friendship and hospitality, for all the nice evenings and for the possibility to relax time and time again in your cheerful world.

- Dominik, for accompanying me through these years, for keeping constant interest in my work and all the laboratory ups and downs. Thank you for sharing my happiness in the good moments and being always at my side in the difficult phases.

- Mam and Dad for your constant support. Thank you above all for your love, but also for providing me again and again with nice food and for sharing perfect times in Sardinia. There is no better place for me to recharge my batteries. Thank you Dad for sparking my interest in nature, it brought me towards many fascinating things, and Mam for teaching me joy and gratefulness over small things.

Many thanks to all who contributed in one or the other way, thank you for accompanying and supporting me, I'm happy to write now these acknowledgements !



Curriculum vitae

Particulars

Name	Christina Maria Ramel
Date of birth	5th September 1978
Citizen of	Gretzenbach SO

Education

1986-1992	Primary school Lostorf SO
1992-1994	Junior high school Kreisschule Mittelgösgen
1994-1999	Grammar school Kantonsschule Olten
January 1999	School-leaving exam type C
2001-2005	Undergraduate studies in biological Sciences University of Zurich
August 2005	Bachelor of Science UZH Biology
2005-2007	Diploma thesis in the group of Prof. Dr. Robert Dudler at the Institute of Plant Biology at the University of Zurich entitled 'The Contribution of Quorum Sensing to the Regulation of Syringolin Biosynthesis in *Pseudomonas syringae* pv. *syringae*'.
March 2007	Master of Science UZH Biology, Microbiology
January 2008 - current	PhD thesis in the group of Prof. Dr. Robert Dudler at the Institute of Plant Biology at the University of Zurich